UNIVERSITY
OF TASMANIA

Understanding Thermophilic Spore-forming Bacteria in Milk Powders

by

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Master of Biotechnology Studies

A thesis submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy

University of Tasmania

April 2017

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List of Abbreviations

× g	times gravitational acceleration
a_w	water activity
ATCC	American Type Culture Collection
BHIB	Brain Heart Infusion Broth
D-value	decimal reduction time
CFU	Colony Forming Unit
CIP	Clean in Place
DETA NONOate	Diethylenetriamine NONOate
DNA	Deoxyribonucleic Acid
EPS	Exopolysaccharide, or extracellular polymeric substances
ESEM	Environmental Scanning Electron Microscope
HACCP	Hazard Analysis and Critical Control Points
H-NOX	Heme Nitric Oxide/Oxygen
MPD	Maximum Population Density
NaOH	Sodium hydroxide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NB	Nutrient Broth
NO	Nitric oxide
NOS	Nitric oxide synthase
O_2	Oxygen
OD	Optical Density
PCR	Polymerase Chain Reaction
QS	Quorum Sensing
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
rpm	Revolutions per minute
RSM	Reconstituted Skim Milk
SC	Spore Count
SEM	Scanning Electron Microscope
SMP	Skim Milk Powder
SS	Stainless Steel

TS	Total Solids
TSA	Tryptone Soya Agar
TSB	Tryptic Soy Broth
TVC	Total Viable Count
v/v	volume/volume
w/v	weight per volume
WMP	Whole Milk Powder

Acknowledgements

I would like to express my sincerest gratitude to the following people for their contributions and encouragement throughout my PhD studies:

i) my supervisor Tom Ross and co-supervisors Mark Tamplin, John Bowman, and Lyndal Mellefont for their help and guidance;
ii) Geoffrey Gardiner Dairy Foundation for financial support for the project *via* Dairy Innovation Australia Ltd., and South Sulawesi Province for my scholarship;
iii) DIAL project manager, Dr. Ian Powell and his colleague, Dr. Barbara Meurer (Milk Quality & Biotechnology Manager of DIAL) for their support and advice and ideas during the project;
iv) Mr. Geoffrey Knight and Dr. Heather Craven from CSIRO Animal, Food and Nutrition Sciences for dairy plants tours, ideas, and assistance
v) Ms. Laura Van de Wardt (2013 intern student at FSC) for permission to use data from her studies on the effect of water activity on *G. stearothermophilus* growth;
vi) Dr. Karsten Goemann of the Central Science Laboratory, University of Tasmania (UTas), and Dr. Warwick Gill of Tasmanian Institute of Agriculture (TIA), School of Land Food (SLAF), UTas, for scanning electron microscopy support;
vii) Honorary Research Professor David Ratkowsky and Dr. Ross Corkrey both of SLAF/TIA, for assistance with data analysis, statistical insights and mathematical modelling support in particular Bayesian analysis provided by Dr. Corkrey
viii) Emeritus Professor Tom McMeekin for suggesting useful research articles;
ix) Associate Professor Scott Rice of School of Biotechnology and Biomolecular Sciences, University of NSW Centre for Marine Bio-Innovation for support on the NO experiments;
x) postgraduate student colleagues and Food Safety Centre staff (particularly Ms. Michelle Williams and Dr. Alison Dann) for providing support and assistance during the long experimental 'runs' using the flow through chamber and for support and also Dr. Brent Seale, Dr. Shane Powell, Dr. Jay Kocharunchitt, Dr. Anthony Baker, and Dr. Mandeep Kaur for mentoring
xi) Mr. Peter Dove, Senior Technical Officer, Central Mechanical Workshops, UTas, for advice in the design, construction, and refinements to the 'flow through chamber'
xii) administrative and technical staff of the School of Land and Food (UTas) for assisting in setting-up experimental systems and support in the administration of

my candidature, including Ms. Lauri Parkinson, Mr. Andrew Measham, Ms. Chantal Woodhams, Ms. Kathryn Ambrose, Ms. Karen Devine and A/Prof. Aduli Malau-Aduli, and staff of UTas' Faculty of Science, Engineering and Technology including Ms. Mandy Fenlon, Ms. Felicity Wilkinson and staff of the UTas' Office for Graduate Research including Ms. Alison Nowak, and Mr. Ben Millett.

xiii) Other postgraduate students for their collegiality, encouragement and support including Ms. Bianca Porteus, Dr. Kevin Feng, Dr. Peipei Zhang, Mr. Ibrahim Abujabhah, Dr. Sarah Zulfakar, Dr. Kamarul Zarkasi, Mr. Tzu Kwan, Mr. Wossen Mengensha, Ms. Akhikun Nahar, Mr. Raihan Mahmud, Mr. Syed Shah, Mr. Tewodros Abebe, Ms. Mona Alharbi, Ms. Novalia Rachmawati, Mr. Radestya Triwibowo and Ms. Kaniz Mohsina;

xiv) my housemates and friends in Hobart, Devonport, & Melbourne (Colin, Nesh, Esther, Bowie, Pru, Mao, Belle, Ayna, Mokhaled, Hilde, Matt, Riddhi), my friends in the Indonesian society (Rifka, Airin, Alin, Arom, Ardi, Budi, Imel, Iskandar, Wazier, Nanda, Randy, Tri, Roby), at DTS Melbourne and Has & Jessie in Melbourne, Fonterra, all of whom became my extended family during PhD study.

No acknowledgment would be complete without expressing my thanks to my parents and sisters. My parents have instilled many admirable qualities in me and given me a good foundation with which to meet life. They've taught me about hard work and self-respect, about persistence and about how to be independent. Mom, especially, was a great role model of resilience, strength and character.

Last, but certainly not least, I acknowledge with tremendous and deep thanks my best friend, Ali Al-Naseri. Our friendship began when we entered the PhD Program in Food Safety at University of Tasmania in August 2011. We immediately became friends and have remained so ever since. Our relationship became deeper four years ago and since then I have been happier, calmer, and more content. Through his love, patience, support and unwavering belief in me, I've been able to complete this long journey.

Abstract

Thermophilic spore-forming bacteria such as *Geobacillus* spp. are common contaminants of milk powder processing plants. Their spores can remain viable throughout the entire dairy powder industrial process, including pasteurisation (72 °C for 15s) and the even hotter evaporator sections. *Geobacillus* spp. can form biofilms in dairy processing equipment that, over extended run times (e.g., >16 h), can deposit unacceptably high spore loads in end-product. While not dangerous to human health, this potentially leads to quality defects and price reductions, requiring that the process be stopped and the plant fully cleaned.

The objective of this study was to:

a. investigate the kinetics of *Geobacillus* growth, biofilm development and spore formation as a function of temperature and water activity,

b. to develop an understanding of how these factors influence thermophile attachment and biofilm formation on stainless steel and affect the time before spore release into milk being processed into milk powder, and, based on this knowledge,

c. explore options to extend run times of dairy powder plants

Growth studies were undertaken, and a stainless-steel, laboratory bench-scale flow-through reactor was built and used, to investigate:

1. The effect of temperature (45 to 75 °C), media composition and water activity (0.959 to 0.992) on growth rates of 16 *Geobacillus* spp. originally isolated from milk powder processing plants;
2. the kinetics of attachment, biofilm formation and eventual release of new spores from spores inoculated into the flow-through reactor system *via* milk and with regard to spore inoculum levels, milk flow rates and temperature;
3. the effect on *Geobacillus* spp. growth of temperature step changes in the flow-through-system, and
4. the potential to disperse biofilms on stainless steel surfaces in the flow-through reactor using exogenously provided nitric oxide (NO).

Growth rates of the 16 strains of *Geobacillus* spp. were modelled as function of temperature using a four-parameter square-root ('Ratkowsky') model. The model was developed to predict the growth of *Geobacillus* spp. under time-varying temperature conditions and to identify temperatures optimal for growth and biofilm formation. Over 300 growth curves were generated at temperatures in the range 45 to 75 °C, using different incubation methods, enumeration methods and growth media, although not all data sets were used because many were deemed to be unreliable due to insufficient or erratic growth of the spore-forming thermophiles under apparently well-controlled growth conditions, a phenomenon reported anecdotally by others. The studies showed that growth occurred in the temperature range 45 to ~70 °C with fastest growth occurring at ~60 °C. Consistent with published reports the generation time at 60 °C was estimated to be ~ 22 – 25 min. Studies at different water activities (a_w) suggested that the minimum water activity for

growth was ~0.975. The results also showed that growth rate variability of all 16 strains is large compared to growth rate variability reported for non-spore forming cells. Nonetheless, no strain had growth rates that were systematically different to that of the pooled data. Growth rates observed for *G. stearothermophilus* W14 were representative of the average response of all strains and this strain was selected for use in subsequent studies.

Growth rate data of *G. stearothermophilus* strain W14 under either anaerobic (100% N_2) or aerobic (20% O_2) conditions were also generated using a bioreactor (fermenter apparatus) at 55 °C, 60 °C, 65 °C, and 70 °C. The bioreactor was employed mainly to enable a comparison of aerobic and anaerobic growth rates by reliably producing anaerobic conditions. *G. stearothermophilus* strain W14 showed fastest growth at ~60 °C in anaerobic conditions with a doubling time of 26 min for vegetative cells, which was similar to growth rates under analogous aerobic conditions.

The kinetics of cell and spore attachment to stainless steel were studied by inoculating the flow-through system with spores of *G. stearothermophilus* W14 in the milk flowing through the system and monitoring the change over time in vegetative cells and spores in the milk leaving the system (i.e., the 'effluent'). Studies were conducted at temperatures from 45 to 70 °C. At near optimal temperatures (*i.e.*, 60 – 65 °C) viable cell and spores counts initially decreased in the milk effluent but began to increase consistently after 3-6 h indicating attachment, germination and proliferation of cells and production of spores. Different milk flow rates applied to the system (5, 10, 20, 40 mL/min) showed no significant differences in the time for the generation of vegetative

cells or spores. Spore inocula, fed into the system as pulses (1 h) or continuously added (usually for ~24 h but up to 40 h in some experiments), showed significant differences in the time to attachment and detectable proliferation, and as a function of temperature. In the pulsed system, the spore counts increased above the inoculum level after ~8 h of milk flow at near optimal temperatures, whereas in the continuous system levels in the effluent milk increased after ~4 h. In the pulsed system, a lower spore inoculum fed to the system (<10^2 CFU/mL), resulted in a longer time before spore counts increased in the effluent (>8 h). Swabbing internal surfaces of the flow-through equipment at the end of “runs” at 45 °C showed early stage biofilm growth (>10^3 CFU/cm^2) whereas runs at 65 °C showed high counts (>10^8 log CFU/cm^2).

The flow-through system was a useful way to study, under ‘commercially-relevant’ conditions, the attachment, growth and sporulation of thermophilic spore formers on warm stainless steel surfaces in milk powder plants and provided a system to study potential interventions against biofilm formation by thermophilic spore-forming bacteria. Based on Knight et al. (2004), who reported a method to minimise biofilm build up using temperature cycling to interrupt the growth cycle of non-spore forming bacteria in whole milk processing, temperature cycling studies were undertaken using the flow-through system to evaluate whether powder plant run times could be extended using the same approach. A number of experiments were conducted with temperature of the system altered systematically during the “run”. In some runs, there was reduced attachment and outgrowth of thermophilic spore-forming bacteria, suggesting that temperature cycling

could extend run times if applied at the sites of most rapid attachment, growth and biofilm formation. The result showed that the temperature cycling should include temperatures near the limits of the temperature growth range of *G. stearothermophilus* to be able to significantly retard growth. Together with the temperature model, these data can be used as a foundation to estimate the expected benefits of manipulation of temperatures of milk powder processes.

Nitric oxide (NO) has been reported to disrupt bacterial biofilms. Its potential for use to disrupt *G. stearothermophilus* biofilms in milk processing equipment was also studied using the flow-through system. At realistic and commercially relevant levels, NO did not significantly delay the time for unacceptable spore levels to occur in the milk effluent, although there was evidence of a reduction in *final* spore loads. However, recent studies have suggested that NO, while effective against Gram-negative biofilms, will not be ineffective against spore-forming Gram-positive bacterial biofilms. In short, the application of NO to extend run times is not supported, however, by the results of this study.

Considering the ecology and physiology of *G. stearothermophilus*, and related species, the results of this study have reinforced that thermophile contamination during dairy powder processing will likely continue to be a difficult problem to address. This is because of the ability of spores to survive processing and cleaning, the rapid growth rate of the microorganism, its ability to form biofilms, and the inevitable production of spores in biofilms. Cell differentiation, including spore-formation, occurs through quorum sensing (QS) mechanisms and appears to be a ‘bet-hedging’ mechanism. It is concluded

that thorough sanitation procedures will still be required at the end of each processing session to minimise residual fouling on stainless steel because the time to unacceptable spore loads also depends on initial contamination levels. While temperature cycling produced some effects, under some circumstances, further research is required to determine whether this approach can be manipulated and optimised to achieve commercially significant extension of powder plant run times.

1 Literature Review

1.1 Introduction

Australia produces a range of dairy products including milk, milk powder, yoghurt, butter and cheese. Milk processing systems are different throughout the dairy food manufacturing sector due to different market requirements, scale of dairy farm operations, and cost. In the case of milk powder, Australia produces different types of milk powder, including whole milk powder (WMP) and skim milk powder (SMP). Besides being a convenient shelf-stable form of 'milk', milk powder is frequently used as a food ingredient and is an important export product.

The quality of milk powder is usually determined by the needs of the commercial customer. There has been an increasing number and stringency of criteria for milk powder, and levels of bacterial endospores less than 10^3 CFU/g are a common requirement (ICMSF, 2011; Hill and Smythe, 2012; Kotzekidou, 2014) with the specification dependant on the intended use of the product and subsequent processing conditions, e.g., if the milk powder will be an ingredient in another product (ICMSF 2011; Hill and Smythe, 2012) higher standards may be appropriate. However, the occurrence and proliferation of thermophilic spore-forming bacteria within the milk powder manufacturing process represents a challenge for dairy industry due to the ability of the thermophilic endospore-forming bacteria to survive processing and grow in association with production equipment rather than inherent contamination of raw milk and survival during processing.

Numerous studies and research projects have sought strategies to overcome the milk powder endospore problem by, e.g., attempting to eliminate contamination and developing better endospore detection tools, to consistently meet market requirements or identify and discard non-conforming batches. However, the problem of thermophilic spore-forming bacteria in dairy powders persists and is costly to producers. Improved knowledge on how to manage the contamination and growth of thermophilic spore-forming contaminants within dairy powder manufacturing systems would help maintain delivery of high quality milk powder.

This review describes the ecology and physiology of endospore-forming thermophilic bacteria that are found in milk powders and also examines the problems they cause in the manufacture of milk powder. The review also assesses how these thermophiles, especially *Geobacillus* spp., contaminate milk powder and also discusses the current systems in place to mitigate the problem.

1.2 Characteristics of thermophilic spore-formers

Endospore-forming bacteria ("spore-formers") that are found in dairy products mainly belong to the genus *Bacillus* (Molva et al., 2009) including *B. licheniformis, B. coagulans, B. pumilus, B. sporothermodurans* and *B. subtilis* (Crielly et al., 1994; Flint et al., 2001; Ronimus et al., 2003; Scheldeman et al., 2006) as well as related genera such as *Geobacillus, Anoxybacillus*, and *Clostridium* (Chen and Hotchkiss, 1993; Aureli et al., 2011). Thermophilic spore-formers that contaminate milk powders typically are

species of the genera *Geobacillus* and *Anoxybacillus*, and *Bacillus licheniformis* (Rueckert et al., 2006; Scott et al., 2007). Spore-formers that contaminate milk powder and dairy environments are not dangerous to human health; however they cause serious contamination problems in the milk industry (Blake and Weimer, 1997; McKillip, 2000; Logan, 2012). They are known to produce highly heat-resistant spores and have the ability to produce extra-cellular enzymes that cause deterioration of reconstituted powdered milk products. Some species are able to degrade hydrocarbons and cellulose and produce enzymes of industrial interest, including amylases, proteases, and lipases. Thermostable enzymes allow these microorganisms to grow from ~50 to ~80 °C (Dheeran et al., 2010).

In the dairy industry, thermophilic bacilli can be divided into two main groups: facultative thermophiles (also known as thermotolerant microorganism) and obligate thermophiles. The obligate thermophiles grow optimally at approximately 48 to 60 °C and mainly include *G. stearothermophilus* and *A. flavithermus* (Flint et al., 2001; Ronimus et al., 2003; Scott et al., 2007; Burgess et al., 2010). *Geobacillus* species were formerly classified in the genus *Bacillus* but 16S rRNA gene sequence data indicated they formed a distinct genus (Nazina et al., 2001). The taxonomy of *Geobacillus* and related thermophilic spore-forming genera *Anoxybacillus*, *Aeribacillus* and *Caldibacillus* was revised by Coorevits et al. (2012) such that *Geobacillus* currently contains 20 valid species and a further four sub-species (LPSN, 2016).

One of the most commonly encountered milk powder contaminants is the species *G. stearothermophilus*, which is the type species of the genus. This species has a reported temperature growth range of approximately 40 to 70 °C (Nazina et al., 2001; Rueckert et al., 2005b; Coorevits et al., 2012; Logan, 2012). The species is able to grow facultative-anaerobically in de Man, Rogosa and Sharpe broth, yeast extract, Nutrient Broth ('NB', Smerilli et al., 2015) and Luria Broth ('LB'; Wu and Welker, 1991). The temperature growth range and production of endospores enables them to survive milk pasteurisation, germinate and grow in product held at ambient or warm temperatures, such as in hot drink vending machines, thereby producing enzymes, acids, and other metabolites that can produce off-flavours in milk. The decimal reduction time or ‘D-value’, which is the time needed to reduce the size of the treated population by a factor of 10 at a particular lethal temperature or under defined lethal conditions, can be used as a measure of the microorganism's or spore's heat resistance. The spores of *G. stearothermophilus* have reported D-values as high as 16 min at 121.1 °C, (Brown, 2000), and 29.9 min at 95 °C under high pressure CO_2 treatment (Watanabe et al., 2003), and they have often been used in sterilisation process evaluation studies because of their high heat resistance.

Also found as spore contaminants in milk powders are strains of *G. kaustophilus* which can grow in Tryptic Soy Broth (TSB) and Luria Broth at a temperature ranging from 45 to 80 °C (60 °C optimum) (Nazina et al., 2001) and are able to produce various thermostable proteins (Suzuki et al., 2012; Suzuki et al., 2013). This microorganism is also reported to grow

anaerobically (Takami et al., 2004a, Takami et al., 2004b); which is contrary to the observations of Dheeran et al., (2010) who grew *G. kaustophilus* aerobically using arsenate in minimum medium supplemented with 0.2% sucrose as the carbon source.

G. thermoleovorans exhibits growth at least from 50 to 70 °C (optimum: 55 to 65 °C) (Nazina et al., 2001; Dinsdale et al., 2011) and is able to produce several thermostable hydrolases including protease, amylase and different esterases (Abdel-Fattah and Gaballa, 2008). These enzymes are stable from pH 3 to 10 and have optimal stability at 70 to 80 °C (Novotny and Perry, 1992). *G. thermoleovorans* is able to grow aerobically at 60 °C in nutrient broth (Graham et al., 2006).

The types of species and their populations found in dairy plants seems to vary (Bylund, 1995) and likely depends on several factors: i) the milk collection season; ii) the composition of the raw milk processed; iii) how well sanitary conditions are maintained during handling and raw milk transport; iv) treatment conditions throughout the milk processing pipeline and, finally, the v) specific operational procedures used in individual factories (Bylund, 1995). *Geobacillus* and relatives accumulate and survive in soil due to their durable spores. The genus grows in soils heated by the sun and in composting soil. They subsequently have been distributed globally on dust (Marchant et al. 2011). The origin of the spore formers as the contamination source thus can be found in farm environments and in raw milk in which dust is deposited and so *Geobacillus* is readily transferred into processing plants on a continual

basis (Crielly et al., 1994; Coorevits et al., 2012). Moreover, food ingredients i.e., sugar, spices, starch, cocoa powder (Lücking et al., 2013) are also likely contributors as contamination sources.

Identification and characterization of spore-former contaminants can be useful information for dairy food processors providing a starting point for appropriate sanitation methods.

1.3 Manufacturing process & contamination

In general, the manufacturing process for skim milk powder consists of four stages: (a) separation of raw milk and pasteurisation (72 °C for 15 s) to eliminate harmful vegetative microorganisms, (b) a preheating step to improve certain functional characteristics of the powder, (c) concentration of preheated skim milk to about 45% total solids (TS) by evaporation of water under reduced pressure, and (d) spray drying of the concentrate to remove most of the remaining water (Papademas and Bintsis, 2010). Typical milk powder manufacturing processes are depicted in Figures 1.1, 1.2 and 1.3). During the manufacture of milk, raw milk is collected, transported, and stored at 5 °C. The source of microbial contamination found in raw milk is usually from cows and the surrounding farm environment. Contamination in milk may also occur during the transportation, storage, transfer (*via* pipelines) and even during packing due to use of contaminated packaging materials (Van Heddeghem and Vlaemynck, 1992). Commonly found microorganisms isolated from raw milk and detected from surfaces of farm milking machines, bulk tanks and dairy silos are *Bacillus* species, especially *B. cereus,*

B. pumilus, B. licheniformis, and *B. subtilis* (Sutherland and Murdoch, 1994; Slaghuis et al., 1997).

Skim milk powder is manufactured using different preheating and concentration methods and temperatures (Mistry, 2002). The condensation temperature is kept in the range of 66 to 77 °C whereas pre-heating may be increased to 94 °C. Pre-heated milk is then transferred into an evaporator where the temperature ranges from 45 to 75 °C (Flint et al., 1997; Flint et al., 2001; Parkar et al., 2003; Scott et al., 2007; Brooks and Flint, 2008; Burgess et al., 2009), which is highly suitable for the growth of thermophilic spore-formers. Homogenisation of milk is also completed before it goes to the evaporator and then to a spray dryer. In spray dryers temperatures range from 120 to 125 °C (inlet air temperature) and 75 to 80 °C (outlet air temperature), (Shrestha et al., 2007).

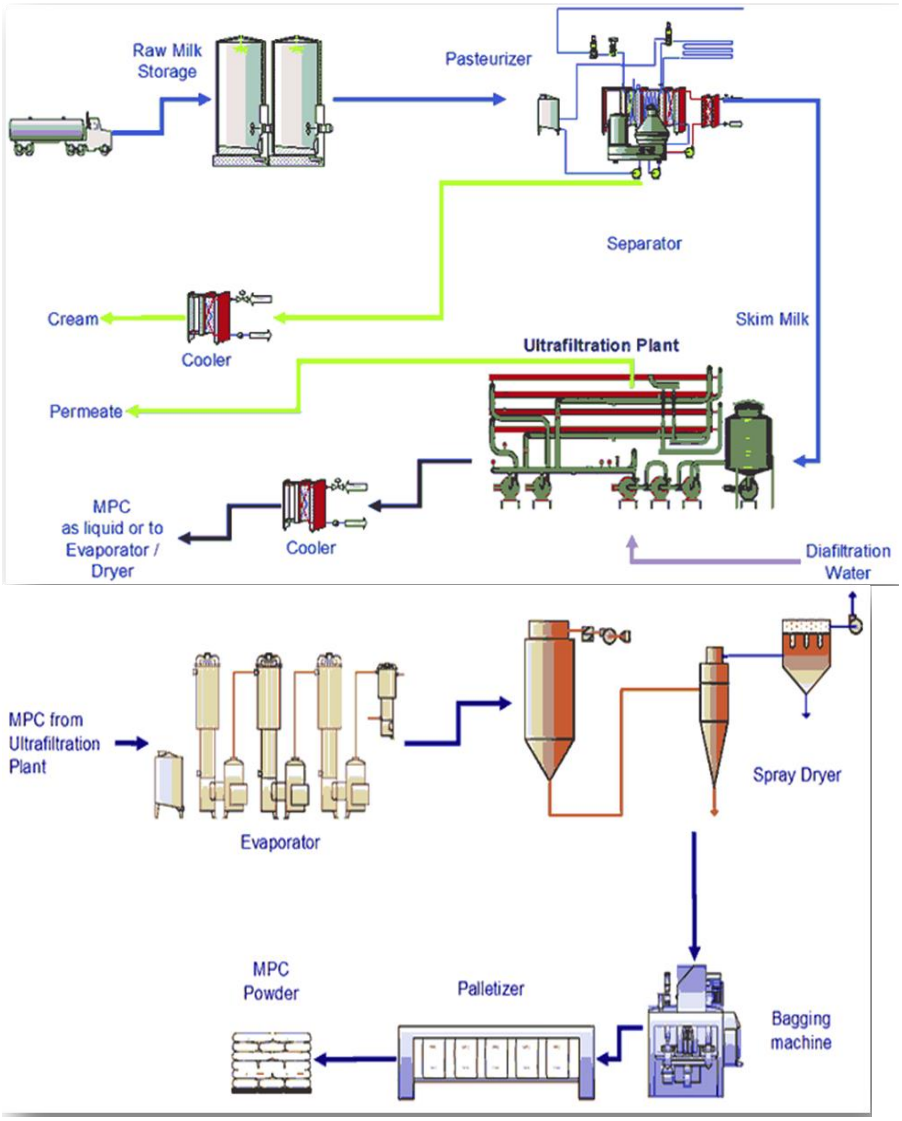


Figure 1. 1 Diagram of a typical milk protein concentrate production process from raw milk receival to final powder. (Reproduced from: http://www.geafiltration.com/library/milk_protein_concentrate.asp)

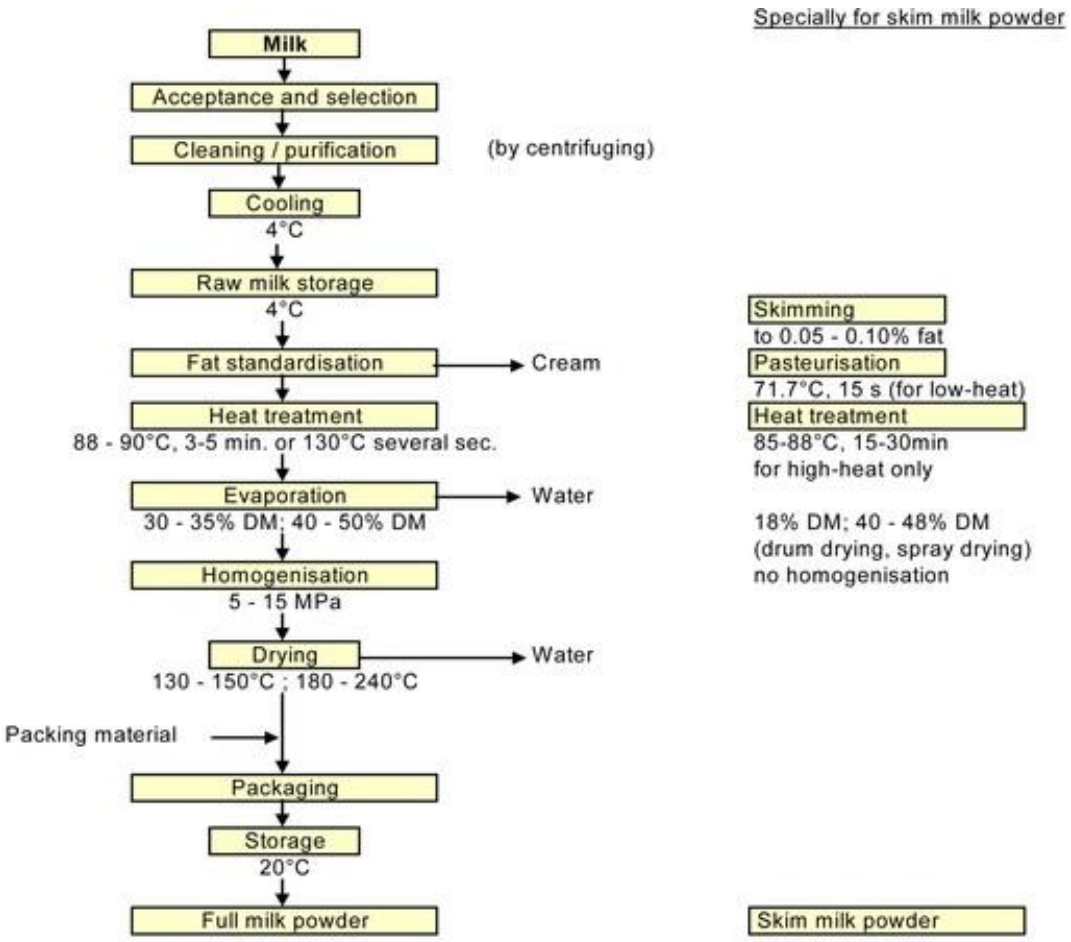


Figure 1. 2 Flow chart showing the process steps involved in full and skim milk powder production (Reproduced from Mistry, 2002).

The increasing number of bacteria found in pasteurised milk as 'run times' extend may arise due to their growth on the wall of the pasteuriser, including the ability to attach to the walls of the downstream sections of the regenerator, reaching final concentrations of approximately 10^6 cells per mL. Thermophilic or thermo-tolerant microorganisms readily attach and grow on or within milk deposits that foul pasteurisers and heat exchangers (Bouman et al., 1982). The cells that attach on the surface may increase in numbers over time as well as degrading the milk during processing (Austin and Bergeron, 1995;

Flint et al., 2001; Oulahal et al., 2008). Milk contains nutrients that support the growth of thermophilic microorganisms that are already attached on surfaces.

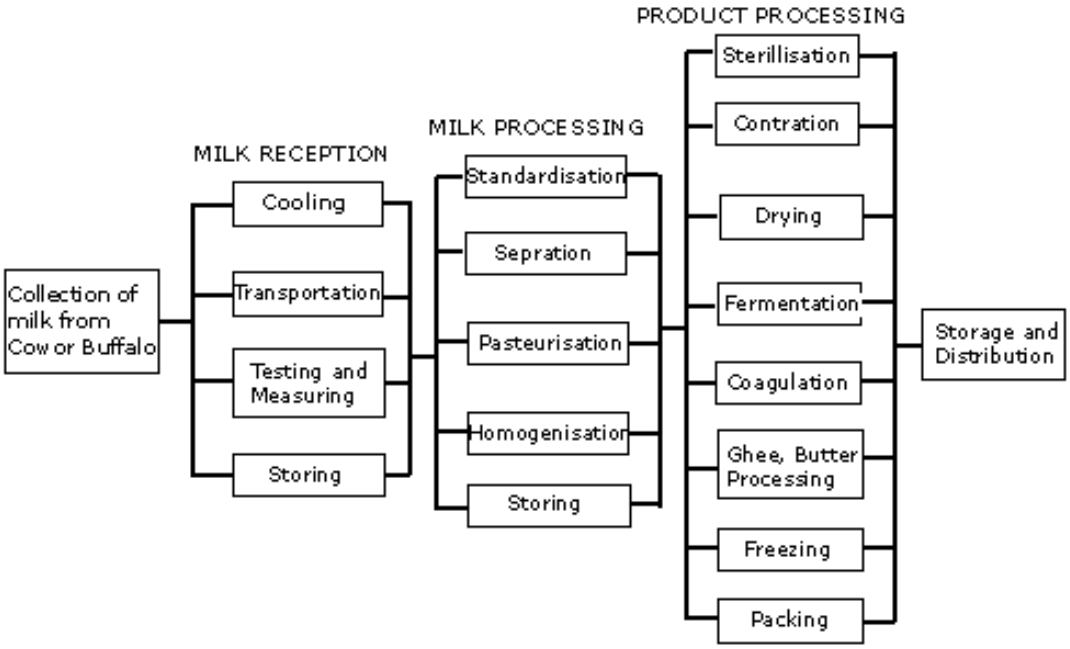


Figure 1. 3 Generic flow chart for diverse milk product manufacture (Reproduced from http://www.powerglob.com/industry/food-industry/281).

Thermophilic bacteria tend to increase in number during milk powder manufacture, especially where there is continuous use of equipment and where subsequent sanitation is not performed properly. Thermophilic bacterial growth can occur in pasteurisers, and evaporators, because of the temperatures at which they operate. In a typical processing run, typically 16 to 22 h, thermophilic spore-formers are able to form biofilms that can protect both spores and vegetative cells during subsequent cleaning (Scott et al., 2007). Eventual dispersal and dislodgement of biofilm cells, including spores,

can contaminate end products, even after cleaning has been performed. Endospores may also be produced in cooler stages of the preheaters and the evaporators (Burgess et al., 2009). Moreover, long production times may permit the growth of cells from lag phase to the stationary growth phase and this may promote dispersal into the process stream. The number of spores from thermophiles, such as *A. flavithermus* occurring in powdered milk typically begins to increase significantly to unacceptable levels after 18-h run times (Brooks and Flint, 2008). The increase in spore number affects the quality and economic value of milk powders produced, and represents a concern for manufacturers owing to the possibility of product spoilage (Lewis and Deeth, 2009).

Although dairy industries rely on pasteurisation at 72 °C for 15 s to kill any harmful, vegetative, microorganisms present in milk and dairy products (Lewis and Deeth, 2009), bacterial endospores remain fully viable and survive throughout the process, since they can tolerate high temperature, acidity and desiccation (Scott et al., 2007; Brooks and Flint, 2008; Burgess et al., 2009). Spore-forming bacilli that are found in milk powders such as *A. flavithermus* and *G. stearothermophilus* are hard to eliminate by pasteurisation (Burgess et al., 2009; Lewis and Deeth, 2009; Burgess et al., 2010).

Owing to the above situation, strategies are needed to prevent or control growth of thermophiles in pasteurisers and evaporators. Currently, the most effective strategy is to process milk for short runs only, and to clean and disinfect the pasteuriser and evaporator equipment between each run

(Bouman et al., 1982). Knight et al. (2004), however, demonstrated that temperature cycling may actually reduce vegetative thermophile growth along the manufacture sections.

1.4 Biofilm formation within milk powder plant systems

Biofilms can be defined as a matrix made from a group of planktonic (free-swimming) microorganisms and their associated exudates that have typically attached to a surface, multiply, and then slough off at various times when conditions are favourable. Characklis (1981) and Stoodley et al., (2002), reviewed biofilm formation and noted that it can be considered to occur by at least four stages: (a) reversible attachment, (b) irreversible attachment, (c) maturation, and (d) detachment. Furthermore, the biofilm structure is influenced during biofilm growth by production of 'Extracellular Polymeric Substances' (EPS), which support the structure. In biofilm formation the matrix, which holds bacterial biofilms together, is a complex mixture of macromolecules including exopolysaccharides, proteins and DNA (Sutherland, 2001).

Besides EPS, in an aqueous environment the interaction between inorganic and organic substances present in that liquid also influences biofilm formation. The absorbed layer, called the conditioning film can alter the charge, hydrophobicity and the free energy of the substratum resulting in microbial attachment. The conditioning film can be formed from collection of protein (milk or whey protein) found in the dairy processing plants (Fletcher and

Floodgate, 1973; Fletcher and Pringle, 1985; Fletcher, 1996; Jeurnink et al., 1996), onto the milk surface.

Within milk powder plant equipment there are several aspects that influence the growth of biofilms that makes them of relevance to the milk industry. These include i) the type of thermophilic microorganism species attaching to the surface of stainless steel; ii) the actual manufacturing process; iii) time length and temperature applied during the process before cleaning takes place; iv) the grade and surface properties of the stainless steel used (Holah and Thorpe, 1990; Brooks and Flint, 2008); and v) the effectiveness of cleaning in place (CIP) procedures including the type of chemical sanitiser used (Christensen et al., 1990; Morton et al., 1998).

1.4.1 The nature of the biofilm formation

1.4.1.1 Type of microorganisms involved

The nature of the biofilm formation depends on the type of microorganisms involved, because they can have different biofilm formation capabilities. The thickness and density of biofilms differs widely between microorganisms ranging from complex layers with differentiated features to simple sparse monolayers. Despite this the degree of biofilm formation and speed of formation crucially depends on the medium it occurs within; availability of nutrients, and on the prevailing environmental conditions, such as the temperature and the speed of the liquid flow rate to which the biofilm is exposed.

1.4.1.2 Shear flow conditions

Vieira et al. (1993) showed that within a continuous flow system the thickness of biofilm is affected by shear stress from the fluid flow rate. The thickness of formed biofilm is also influenced by nutrients and substrates within its environment, such as carbon, nitrogen and oxygen. Starvation of biofilm cells may weaken the attachment of formed biofilms hence cause a greater amount of sloughing off from the surface (Christensen and Characklis, 1990; Stoodley et al., 1999). Similar observations were made by Chang et al. (1991) and van Loosdrecht et al. (1995) within a biofilm airlift suspension reactor to examine biofilm formation under different shear flow conditions in water treatment plants. In terms of thermophilic spore-formers in dairy powder plants, Burgess et al. (2010) concluded that such biofilms might only be present as monolayers in areas of the plant that have high shear forces (i.e., product flow), and that are regularly cleaned and where there are no 'dead zones' (of low flow rate). Hydrodynamic forces may affect biofilm structure and lead to detachment from the surface (Picioreanu et al. 1999, 2000, 2001; Chopp, et al., 2002; Purevdorj, et al., 2002,). Equally, however, there are other areas in a dairy powder plant where flow rates are low or inconsistent (e.g., underneath the distribution plates of evaporators) and where multi-layer thermophilic biofilms could form. Factors affecting biolfilm formation in dairy powder plants are further discussed in Section 1.4.2.2.

1.4.1.3 Nutrient availability

Biofilm formation of many other species is initiated by changes in nutrient

availability, for example *P. aeruginosa* and *P. fluorescens* initiate biofilm development in response to a nutrient-rich medium, and return to the planktonic mode of growth when they are nutrient deprived (O'Toole et al., 2000). This nutrient modulated response allows the cells to adapt to fluctuations in nutrient resources.

Zhang et al. (2014) reported that during conditions where carbon sources were at limiting levels, matrix production of biofilms was increased. Similarly, in a study involving the interaction of *E. coli* and *P. aeruginosa* in various media, nutrient limited conditions consistently resulted in increased biofilm growth (Culotti and Packman, 2014).

1.4.1.4 Oxygen concentration

In their work with both *Anoxybacillus* and *Geobacillus,* Zhao et al. (2013) hypothesised that the oxygen concentration may affect biofilm formation on stainless steel surfaces, especially at elevated temperature and in industrial piping systems that are exposed to oxygen during operation. They concluded that *Anoxybacillus* and *Geobacillus* prefer oxygenated environments, being found preferentially at air liquid interfaces in such systems. Oxygen concentration influences the growth of *Anoxybacillus* spp. as facultative anaerobes (Nakano and Zuber, 1998; Pikuta et al., 2000). Rice et al. (2005) reported that the availability of nutrients or oxygen has a great influence on attachment and detachment processes of biofilms.

1.4.2 Fouling in milk processing plants

Geobacillus and *Anoxybacillus* spp. can form biofilms, originating from vegetative cells or spores that have attached to stainless steel equipment surfaces within the processing line (Brooks and Flint, 2008; Burgess et al., 2009; Teh et al., 2014). When milk casein and whey proteins denature, the deposits can lead to milk fouling and may influence biofilm growth and formation (Komlos et al., 2006) especially when temperature increases above 60 °C (Kinsella and Whitehead, 1989). Milk denaturation can happen in any location where the temperature ranges from 25 to 70 °C.

Fouling in milk processing plants is a complex process involving interactions between protein aggregation and deposition, as well as formation and deposition of various crystals of phosphate and calcium species that may also influence protein fouling (Rosmaninho and Melo, 2006). Rosmaninho and Melo, (2006) studied the influence of pH, citrate (a natural component of milk) and temperature to better understand the processes that influence fouling of surfaces in milk processing plants. Fat and protein deposits provide protective effects to various thermophilic microorganisms against CIP procedures (Mattila et al., 1990; Chmielewski and Frank, 2003). Research that seeks to understand fouling and biofilm formation mechanisms in milk powder manufacturing systems will potentially provide important clues for developing means to minimise the formation of milk deposits and mitigation of spore contamination.

Much work has been done to study fouling and biofilm formation in laboratory

environments. McCoy et al. (1981) using a mixed tubular reactor with low and high fluid velocity, showed that when fouling commenced, biofilms start to increase. As in dairy processing lines, membrane biofouling occurs when certain bacteria become attached to the surfaces, which commences the process of biofilm formation (Liao et al., 2004).

When milk casein and whey proteins denature, e.g. in any location where the temperature ranges from 25 to 70 °C, the deposits cause fouling and result in increased biofilm growth and formation, especially when temperature increases above 60 °C (Kinsella, 1989).

1.4.2.1 Sporulation

During biofilm formation and maturation, sporulation may occur, depending on the conditions of the environment. Burgess et al. (2014b) studied 10 strains of *G. stearothermophilus* and one A*noxybacillus* species isolated from a milk powder manufacturing plant and concluded that they showed differential ability to produce spores from biofilms.

Flint et al. (2001) and Burgess et al. (2009) showed that certain thermophilic spore-forming species readily attached and grew on stainless steel surfaces, forming mature biofilms of $>10^6$ cells per cm^2 in only 6 h and reaching a steady state after 8 h.

Variations in the environment including pH affect the viability of the cells

Under favourable conditions highly heat-resistant spores in reconstituted milk

will germinate and then become metabolically active and begin to grow. Spore development and maturation is affected by nutrients including availability of amino acids, minerals, glucose (and other carbon sources), and B complex vitamins (Penna et al., 2003).

Most bacteria, including *Geobacillus*, favour conditions with a near neutral pH (i.e., 7). Conditions that are favourable to spore formation and general vegetative growth are pH (4.5-6.5) with water activity (0.95-1.00) (ICMFS, 2012)). Thermophilic *Bacillus* strains grow in a pH (4.5-9.5) in broth media and able to encourage the attachment on stainless steel (Lindsay et al., 2005) and prefer temperatures in the range 40 to 70°C . Conditions for growth of *G. stearothermophilus* are considered in more detail in Chapter 2 of this thesis.

Therefore, knowledge of heat resistance and the conditions that trigger sporulation and germination are important to manage milk powder production and to limit end-product quality and reconstituted product spoilage. It is also important to determine the duration and temperature of heating within processing lines in milk powder plants to reduce the presence of spores in milk products.

1.4.2.2 Biofilm Development and Dispersal

Cell surface hydrophobicity has an important role in biofilm development and dispersion onto surfaces which greatly affects the composition of the biofilm matrix (Sutherland, 2001). The shear rate will determine the rate of cell removal and regions of the matrix from the biofilm (Donlan, 2002) thus

significantly affecting physical behaviour (Brindley et al., 2011). Because of the matrix's broad range of properties and flexibilities, under turbulent flow, for example, the biofilm's structure changes in shape and at the end will lead to detachment process (Stoodley et al., 1999). Moreover, under higher flow rates, the matrix appears to be more firmly attached (Ruggeri, 2009; Vickery et al., 2009).

The Derjaguin-Landau-Verwey-Overbeek (DLVO) theory helps explain the process of bacterial adhesion to a surface by suggesting it involves a combination of electrostatic repulsion and van der Waal forces to achieve positive surface attraction energy. Katsikogianni and Missirlis (2004) studied this concept by comparing detachment rates in relation to fluid flow against a biofilm thus defining the strength of the interacting forces.

The sloughing off of biofilm material is the main source of spore contaminants in milk powder products (Rueckert et al., 2004). *Bacillus* species spores are strongly hydrophobic and the ability for spores to adhere to glass and stainless steel depends on hydrophobicity, surface charge and overall cell density (Ronner et al., 1990; Grand et al., 2010).

Spores are more resistant to environmental stress than vegetative cells due to their metabolic dormancy and tough physical nature (Jenson et al., 2003). Spores attach to stainless steel more readily and in greater number than vegetative cells (Flint et al., 2001) and cell-surface proteins play an important role in the initial process of attachment during the formation of biofilms (Parkar

et al., 2001). Furthermore spores are reported to be more hydrophobic than vegetative cells (Ronner et al., 1990, Peng et al., 2001, Faille et al., 2002).

There are two adhesion stages recognised: the primary (“docking”) and the secondary (“locking”) phases (Dunne, 2002). In the primary phase, the interaction of the surface with microorganisms floating or actively swimming over the surface depends on hydrophobic interactions, van der Waals forces, temperature, and hydrodynamics (Carpentier and Cerf, 1993). In this phase, attaching bacteria are easily removed, especially by rinsing (Allison and Sutherland, 1987; Marshall, 1992; Poulsen, 1999). Typically: 1) vegetative bacteria prefer to adhere with this mechanism, 2) the adhesion process can be controlled in aquatic systems where nutrients availability is abundant, and 3) biofilm may occur in extremely oligotrophic environments even though attachment to surfaces is difficult. Furthermore, as noted earlier, bacterial adhesion can be influenced by different environmental conditions, including the presence of nutrients and also species-specific attributes of the cell (Costerton et al., 1995; O'Toole et al., 2000).

In the ‘locking’ phase, planktonic microorganisms, which can be different species, may attach to each other and to the surface. Synergies between different microorganisms in attachment processes were reported by Leung et al. (1998) where Gram positive and negative bacteria adhered to each other after production of glycoprotein-polysaccharide.

1.4.2.3 Maturation

The biofilm maturation process happens after bacteria attach to a surface under relatively favourable conditions (O'Toole et al., 2000). The production of exopolysaccharides or other forms of EPS provides biofilm-associated bacteria several advantages including trapping minerals and nutrients from environment (Costerton et al., 1987; Carpentier and Cerf, 1993) and surrounding and protecting attached bacteria on the surface. Figure 1.3 (Costerton et al., 1987) illustrates a biofilm that consists of a consortium of microbes. In natural environments, biofilm growth is initiated and stimulated by primary production (B) where inorganic resources such as light, CO_2 and inorganic minerals generate organic biomass (e.g., algal photosynthesis) that simultaneously leads to a layer of EPS that can trap nutrients flowing past the biofilm (A). The biofilm layer also forms a solid protective layer (D). Bacteria embedded in the biofilm continually (C) digest the substratum which includes passively trapped nutrients thus sustaining the biofilm colony. The primary system produces cell-associated digestive enzymes that produce nutrient substrates that stimulate the continued growth.

According to Flint et al (1997) process biofilms in dairy manufacturing plants differ from biofilms associated with other food processing facilities, or other environments. In dairy plants, biofilms of a single species often predominate, possibly because of the highly selective heat treatment of milk(pasteurisation) which reduces the competition from heat sensitive Gram-negative species and favours the thermoduric species which are more often found in processed milk and milk products. Process biofilms in dairy plants are also characterized by

their rapid development, derived from the fast growth rates of the organisms involved, such as G. stearothermophilus, but also the ample supply of nutrients, warmth and moisture.

The process of biofilm adhesion and cell development suggests that there are cell-to-cell signalling mechanisms (McLean et al., 2005) that regulate or stimulate bacterial growth, metabolic systems and functional communal coordination (Carpentier and Cerf, 1993). Cell-to-cell signalling or quorum sensing (QS) depends on environment conditions (McLean et al., 2005) and the use of chemical signalling molecules such as acylated homoserine lactones (Fuqua et al., 2001) in Gram-negative bacteria. QS systems 'monitor' microbial population density and have been shown to be important in the regulation of the development of biofilms through stimulation of specific gene expression and resultant biochemical pathways in biofilm bacteria.

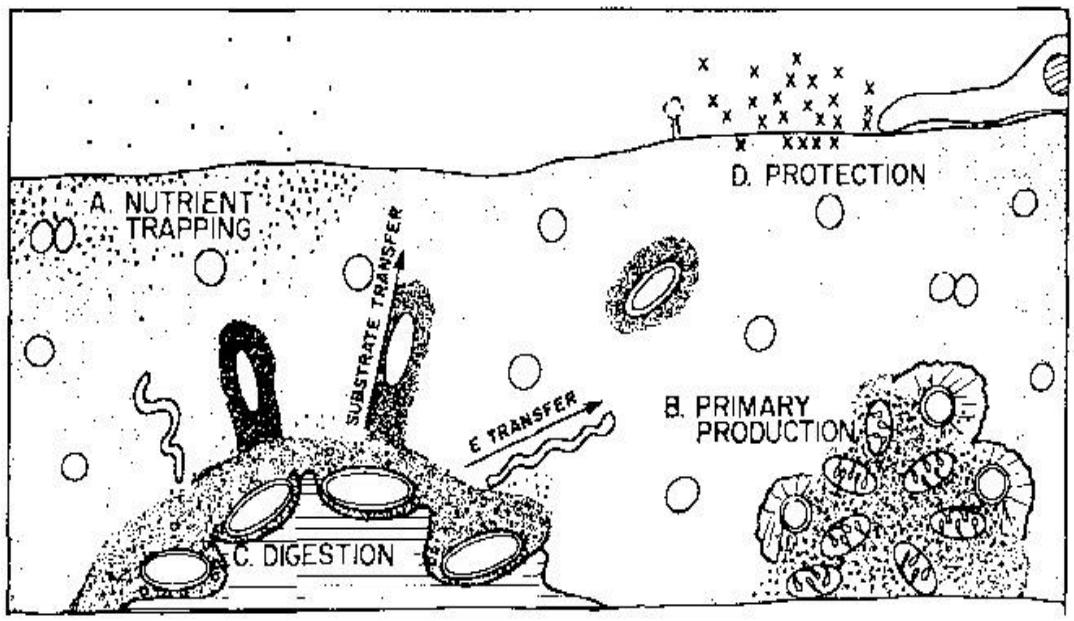


Figure 1. 2 Diagram of biofilm protection mode. (A) Nutrient Trapping (B)

Primary production. (C) Formation of a digestive consortium. (D) Exclusion of antibacterial substances. (Reproduced from Costerton et al., 1987).

1.4.2.4 Detachment

Biofilm detachment occurs when biofilm substances and cells separate from the biofilm due to shear forces including flowing fluid on the surface, abrasion, scrubbing, or any other forces that affect the separation and different ionic conditions during growth (See Figure 1.4) (Pavlovsky et al., 2013).

Biofilm detachment may be initiated by enzyme production that breaks down the EPS layer (Molobela et al., 2010) as well as environments that contain proteases and polysaccharide hydrolases (Lindsay et al., 2000).

Throughout the stages of surface contamination, mechanical stress can be transmitted by the flow of medium (shear stress). Ions such as Ca^{++} and Al^{+++}, along with hydrogen interaction of the cells and medium, affect the biofilm attachment and production of EPS (Sutherland, 1983). Moreover, the presence of shear forces and different ionic conditions such as the availability of Cu^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+} and Al^{3+} during growth, can also effect biofilm mechanics (Grumbein et al., 2014).

When shear forces are generated by flowing liquids, detachment of individual bacteria or erosion of biofilm fragments may also depend on the thickness of the biofilm (Arcand et al., 1994), and this can be demonstrated in fluidised

beds (Chang et al., 1991). As a result, knowledge of the environment, chemical conditions and biofilm species interactions may help understanding of the mechanisms of attachment and detachment of bacteria from biofilms. As illustrated in Figure 1.4 there are various modes of dispersal of biofilm and cells associated with biofilms.

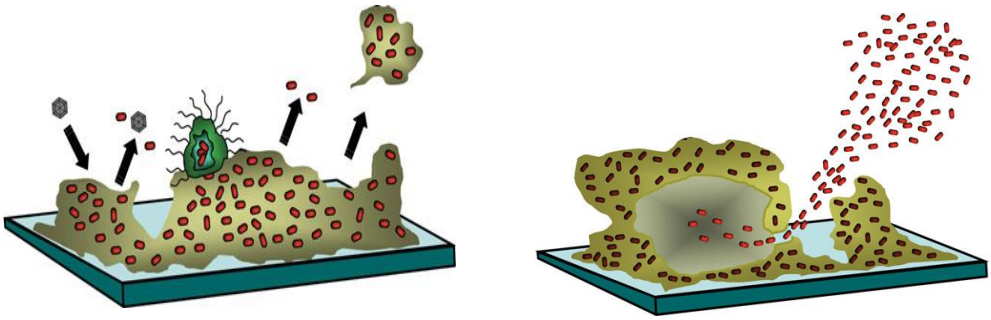


Figure 1.3 Representations of (left) abrasion, grazing, erosion, sloughing, fluid shear movement on biofilm detachment and (right) biofilm dispersion by cells inside the biofilm escaping the biofilm as planktonic bacteria (Reproduced from Davies, 2011).

1.5 Detection methods for thermophiles

The common thermophilic contaminants found in dairy powder processing plants throughout the world include *Anoxybacillus, Geobacillus* and *Bacillus* species. As noted earlier, thermophilic spore-formers that can exist in some parts of dairy powder plants (operating from 40 to 75 °C) and contaminate end products have become a problem that needs to be solved: they are costly to industry because they currently preclude long production run times (Flint et al., 1997; Flint et al., 2001; Parkar et al., 2003; Scott et al., 2007; Brooks and Flint,

2008; Burgess et al., 2010; Burgess et al., 2014b). Numerous approaches to minimise the presence of these thermophiles such as frequent cleaning (Kessler and Lund, 1989; Mattila et al., 1990; Gibson et al., 1999; Parkar et al., 2004; Bremer et al., 2006; Alvarez et al., 2007; Vickery et al., 2009; Simões et al., 2010), short production runs (Murphy et al., 1999; Flint et al., 2007; Scott et al., 2007; Watterson et al., 2014), and intensive quality control (Sandrou and Arvanitoyannis, 2000; Chye et al., 2004; Arvanitoyannis and Tzouros, 2005), have been proposed and implemented to save operating costs and minimise contamination in end products (milk powders). Detection methods for thermophiles from dairy plants are described below.

Thermophiles can be found as spores at several places in powder plants, e.g., in biofilms or the 'biofouling' layers in production lines, on the walls of the processing equipment, or 'dead zones' in pipes. The diversity in growth location, condition, and characteristics of thermophiles, and the sheer scale of milk powder processing plants make thermophiles difficult to 'find' and detect in a specific area of the processing equipment. Thermophiles may grow as biofilms in separators, pasteurisers and evaporators within the range of 45-60°C (Burgess et al., 2010), or at even higher temperatures (see Chapter 2). Other factors that influence the growth is raw milk quality, product type, and the type of stainless steel (Wang et al., 2015). Their importance, however, has already been described above. Therefore, ideally there would be methods that give information about them quickly and inexpensively. Detection methods can include visual detection and microbiological techniques from samples. In this case, thermophiles can be visualised by using a microscopes.

Epifluorescence microscopy can be used to observe biofilms on surfaces (Lichtman and Conchello, 2005). The identification requires the isolation of pure cultures followed by testing for multiple physiological and biochemical tests, such as PCR identification or RAPD fingerprints (Ronimus et.al., 2003), species-specific PCR (Flint et al., 2001), and partial 16S rDNA sequencing.

Ideally, monitoring tools should provide on-line, real-time information about the current and predicted condition and contamination of the system.

1.6 Microbial quantification

Determining microbial concentrations provides information on the ecology of microorganism of interest, to enable monitoring programs to control the growth to be devised, to enable assessment of exposure(s) from the environment. This includes evaluating risk, evaluating the effect of treatment processes and determining if samples meet regulatory, customer or other requirements for microbial quality.

The most common methods used for evaluating microbial numbers include microscope-based cell counting (Rueckert et al., 2005b; Burnett et al., 2006; Watanabe et al., 2008; Mohd Saidi, 2014), colony forming units (CFU) or plate counts (Scott et al., 2007; Palmer, 2008; Burgess et al., 2009; Khanal et al., 2014) and turbidimetric measurements (optical density [OD] at 600 nm) (Burnett et al., 2006; Al-Batayneh et al., 2011; Marchand et al., 2012; Zhao et al., 2013).

Spore enumeration has been done by utilising a heat treatment (80°C x 20 min) to eliminate vegetative cells. Pettipher et al. (1996) found spores of *A. acidoterrestris* could be enumerated by heat treating samples at 80°C for 10 min. They used a microscope and observed 90% of *A. acidoterrestris* spores were recovered by incubation on OSA plates for 7 d at 44°C. Barbeau et al., (1997) investigated 3 different kinds of pasteurisation methods to detect enumerated spore after a heat treatment at 75°C for 15 min followed by culture on plates at 35°C for 24h.

1.6.1 Microscope-based cell counting

The microscopic inspection of samples (with or without the use of dyes) can enumerate bacteria and give information on dead/live cells and the structure of bacteria adhered on a surface. There are many types of microscopy used in cell counting methods such as fluorescence microscopy (Rueckert et al., 2005a; Nocker et al., 2006; Luecking et al., 2013), confocal laser and scanning microscopy (Burnett et al., 2006; Burgess et al., 2009; Marchand et al., 2012), and environmental scanning electron microscopy (ESEM) (Abrusci et al., 2005; Poli et al., 2006; Burgess et al., 2014a).

Direct microscopic counts are usually done using slides called counting chambers. In using this method, dead cells are hard to differentiate from living cells but certain dyes can help to distinguish live cells from dead cells (Nocker et al., 2006). Moreover the cells need to be centrifuged or filtered to achieve a sufficiently dense suspension (>10^7 cells per mL). Proper handling of cultures

is also needed to maintain desirable pH and temperature in growth media to avoid loss of cells. The cell count/concentration in liquid media is usually expressed as a number of cells per unit of volume. The advantage of this method is that it is cheap and provides rapid estimates of cell number.

1.6.2 Plate counts or colony forming units (CFU)

Microbial quantification using plate counts is time consuming (typically takes 1-5 days) and may require skilled technicians. However this technique is inexpensive and provides invaluable microbiological information on viable cell densities in the sample. Disadvantages are that colonies may be derived from clumps of cells, thereby underestimating the true cell count and that the method depends on suitable growth media and conditions that enable colony growth.

This method involves sample dilution and spreading an aliquot of a diluted sample on an agar plate, or utilising newer alternatives such as 'The 3M™ Petrifilm™'. After incubation, each colony is counted in relation to the dilution and expressed as the total number of CFU which represents the viable number of bacteria in the sample.

Samples for enumeration in milk or dairy products can be collected from desired sampling points or, from surfaces, by swabbing. Swabbing can be done where the microorganism is believed to attach, by using sponge or cotton wool depending on the area (Burgess et al., 2009a; Seale et al., 2010; Luecking et al., 2013; Zhao et al., 2013; Burgess et al., 2014a, b). The swabs

are then transferred into a recovery medium for release and resuspension and application of counting methods, such as described above. To measure the strength of bacterial adhesion it is necessary to remove them from a surface (Bakker et al., 2003). Biofilms can be removed mainly by brushing, scrubbing and scraping affected surfaces. Several studies used electron microscopy and atomic force microscopy to examine the structural strength of the spores (Henriques and Moran, 2000, Leuschner and Lillford, 2001). The strength of adhesion may greatly affected by composition of outer layer such as protein and carbohydrates such as a *B. subtilis* spore (Matz et al., 1970, Waller et al., 2004).

Adhesion measurement can be divided into: (a) counting cells before and after a treatment to see the difference (b) applied force during the measurement such as swabbing method to remove cells from the surface for enumeration. Moreover, adhesion can also be investigated using imaging equipment to observe directly the adhesion of bacterial populations. Critical force techniques directly interact with bacteria so that a force required to remove bacteria can be determined (Merz et al., 2000; Garrett et al., 2008).

The biofilm adhesion strength on the surface depends on the fouling materials e.g., milk deposits and surface composition (Liu et al., 2002), as considered in DVLO theories (Gallardo-Moreno et al., 2002). Removal techniques and the measurement of forces required can use several methods such as atomic force microscopy (AFM) (Razatos et al., 1998, Beech et al., 2002, Chaw et al., 2005) and flow cell techniques (Stoodley and Lewandowski, 1994). Flow cell

techniques can be used to observe the mechanical and cohesive properties of biofilms, but cannot be used to measure directly the forces required to remove biofilms from a surface. A Robbins device has been used to provide valuable information on the physiology and metabolism of biofilm bacteria, regulation of various bacterial genes and their products, and resistance to antimicrobial compounds and their interactions with various components of the immune system (Ramage et al., 2008).

A disadvantage of swabbing is that bacteria from the surface may only be partially removed from the surface; therefore the number of bacteria recovered may be less than the actual number present.

1.6.3 Turbidimetry measurement

Turbidimetric methods are appropriate to quantification of cell suspensions in clear liquids. The method is relatively insensitive and relies on measuring turbidity of the sample due to the presence of microbial cells. Turbidimetric methods cannot measure cell concentrations less than approximately 105 to 106 cells/mL, unless large volumes are used in the measurement. The method works by determining the absorption (spectrophotometry) or scattering of light (nephelometry) passing through the sample by the cells in the sample. The higher the cell concentration, the more turbid the suspension, and the greater the attenuation of the light as it passes through the sample.

Spectrophotometers can be used to measure that attenuation. Nephelometry, which measure the light deflected by the sample, rather than its attenuation

(i.e., spectrophotometry), can also be used. The sample is placed in a transparent cuvette, or a micro titre plate (as little as 1 microliter) and the optical density can be measured. Optical density ('OD'), at a given wavelength, or absorbance, can be related to cell concentration or biomass in the cell suspension by reference to calibration curves. Disadvantages of this method are that cells are not counted directly, because the machine measures light scatter or absorbance, not cells, its relative insensitivity, the need for a clear suspending medium and that it provides only an estimation of cell numbers.

1.6.4 Molecular techniques

Quantitative, more sensitive, and quicker procedures are desirable to identify the contaminants in dairy processing lines. Real-time quantitative PCR is a widely used technique in microbial community analysis, especially to quantify the number of target genes, including in samples from dairy plants, based on the time taken to detection, i.e., the number of PCR amplification cycles required prior to obtaining a detectable PCR product. However, the method needs appropriate standards and the availability of DNA/RNA templates for the target microorganism. Although quantitative molecular methods have benefits including, potentially, faster and more specific detection, and the ability to quantify very specifically key microbes of interest, the efficiency and practicality of the method needs to be further developed, i.e., configurations that can be implemented in industry, and without the need for specialist operators and the ability to identify and multiple targets simultaneously, e.g., through the use of multiplex approaches.

Direct sampling of microbial communities can be done by PCR and sequencing-based analysis of DNA samples extracted from reconstituted milk or milk powder samples collected from processing lines and plants. The presence of spore-formers detected and identified by reference to (known) DNA sequences (Fykse et al., 2003; Rueckert et al., 2005a; Masoud et al., 2012; Bottari et al., 2013; Chauhan et al., 2013). To study diversity in ecosystems, in relation to the phylogenetic relation between strains, and to identify and characterise microorganism at species and subspecies levels, techniques such as fatty acid methyl ester and rep-PCR profilings, and 16S rRNA sequencing (Nazina et al., 2001; Adiguzel, 2006) can be used. rep-PCR is used with PCR primers to enable the amplification of DNA fragments and widely used in the characterisation of Firmicutes, Gram negatives and *Actinomycetes* (Versalovic et al., 1993; Masco et al., 2003).

Marker genes used besides 16S rRNA include the sporulation transcription factor *spo0A* that is present in endospore-forming bacteria found in dairy processing lines (Ronimus et al., 2003; Rueckert et al., 2004) and *ITS 16S-23S* rRNA region and the *rpoB* gene sequence (Pennacchia et al., 2014) with primers designed specifically to identify spore-formers.

1.6.5 Modified biofilm growth techniques

Modified biofilm growth techniques are used to simulate actual conditions under which biofilms might grow such as in dairy plants. A number of techniques have been developed, relevant to growth of microbial biofilms in

milk powder processing plants, offering a variety of observations and potential solutions to manage undesirable biofilm growth. For industrial processes, biofilm reactor systems operated in continuous flow mode provide automation, cell uniformity, and economic value. By utilising a reactor, a sample can be drawn in order to study the biofilm adhesion that can be analysed periodically (Purevdori et al. 2002).

There are two main biofilm growth techniques, namely static and dynamic growth. Growing biofilms in a static growth technique is relatively simple compared with dynamic techniques, allowing the culture to grow in media where there are few mechanical or thermal fluctuations (Peng et al., 2002). However, nutrients become depleted over time, affecting biofilm growth. In static growth techniques, various growth assays have been used, *e.g.,* the microtiter plate biofilm assay (Palmer, 2008; Somerton et al., 2012; Burgess et al., 2014b) to assess bacterial attachment by staining, and the air-liquid interface assay (Marchand et al., 2012; Zhao et al., 2013) to provide a mechanism for direct microscopic viewing of the live attached microbes (Merritt et al., 2005).

Dynamic growth methods generally include those that grow biofilms with the use of liquid flow, where forces of attraction between bacteria and surfaces become greater than the shear forces of the flow for biofilm survival (Johnston and Jones, 1995; Schmid et al., 2004). A continuous flow laboratory reactor was used to grow biofilms and determine the rate and the extent of spore formation under controlled conditions by Burgess et al. (2009) and Flint et al.

(2001). Others studies have used a biofilm grown under low shear conditions using the drip flow biofilm reactor and the rotating disk biofilm reactor (Goeres et al., 2009; Schwartz et al., 2010). Furthermore, flow techniques may be used to observe attached cells on surfaces. Chemostat (continuous culture) has been used to study the effects of nutrient addition (Davey and O'Toole, 2000; Schmid et al., 2004).

1.7 Thermophilic spore-formers: Growth management

As discussed, milk powders in dairy plants are prone to thermophilic spore contamination due to the ability of these spores to survive pasteurisation processes. Thermophilic microorganisms are able to form a biofilm (Langeveld et al., 1996), grow and attach to stainless steel with doubling times around 19 min at 55 °C (Flint et al., 2001). The spore number can reach up to 10^4 CFU/mL after activation by heat treatment at 80 °C for 10 min (Rueckert et al., 2004). Although dairy manufacturers are aware of this issue, it is not possible yet to maintain low number of spores in end products, i.e. below 10^2 CFU/g, or to eliminate them.

There have been efforts to extend current run times by more than (an additional) 6 h, for example, by time-temperature cycling (Knight et al., 2004), process configuration and cleaning procedures (Bremer et al., 2006; Alvarez et al., 2007; Tang et al., 2010), or biofouling controls (de Jong et al., 2002). There is a need, however, to ensure that any such processes are performed effectively and to pay close attention to equipment cleaning and sanitation.

1.7.1 Clean-in-Place (CIP)

The CIP process is done to ensure plant hygiene by eliminating most proteins, fats and mineral deposits on processing lines from milk processing at dairy plants after every 18-24 h processing run. The CIP aims to remove biofilm build up on stainless steel along the processing lines (Austin and Bergeron, 1995; Jullien et al., 2003).

Cleaning-in-place (CIP) procedures usually start with cold water to rinse, detergent to remove residues, another rinse of water to remove the detergent, a sanitiser or acid to inactivate and kill any residual microorganisms, and a final cold rinse (Forsythe and Hayes, 1998). The methods for biofilm removal may involve combinations of scrubbing with the use of cleaning agents and sanitisers to remove biofilm layers. However, bacteria may develop resistance against some sanitisers, for example, by producing extracellular polysaccharides (Donlan et al., 2002).

The common sanitisers used to reduce biofilm attachment of *Bacillus* and related species are combinations of hydrogen peroxide and peracetic acid, in working concentration of ~1-2%, combined with a contact time of 5 minutes at 25°C (Caixeta et al., 2012).

A typical CIP process at a dairy plant consists of rinsing with warm water for about 10 min, an alkaline wash (0.5-1.5% sodium hydroxide (NaOH) at 70-80 °C for 30 min) or acid wash (0.5% nitric acid at 60 °C for 20 min)

followed by rinsing out alkaline detergent with warm water for about 5 min (Bylund, 1995). The alkaline treatment removes proteins, fats and carbohydrates (Grima et al., 2003), while the acid dissolves minerals. Sodium hypochlorite is widely used as a disinfectant.

There are also modifications to CIP process to reduce bacterial attachment on surfaces and matured biofilms (Parkar et al., 2004); especially the use of enzyme cleaners (Bremer et al., 2006; Tang et al., 2010), use of bacteriophages (Roy et al., 1993), trisodium phosphate (Somers et al., 1994), oxidizing agents (Meyer, 2003), or antimicrobial agents (Stewart and Raquepas, 1995).

However, the effectiveness of CIP depends on the nature and age of the fouling layer found in certain areas such as dead ends, joints, valves and gaskets (Somers et al., 1994; Austin and Bergeron, 1995), condition of surface that is being cleaned, cleaning agent used (combination of chemical composition and concentration), application time, and temperature (Krysinski et al., 1992, Lim and Bai, 2003, Al-Amoudi and Lovitt, 2007).

Although there are variety of CIP treatments proposed and applied to remove biofilms from a surface, there are few principles to consider in their selection and refinement, such as consideration of how well the biocide used will penetrate the EPS layers to act on cells promote their death and dislodgement, whether timing of disinfection, e.g., before a biofilm is established, and the potential effects of the chemical agents on equipment

and end products (Meyer, 2003).

1.7.2 Nitric Oxide

Nitric Oxide (NO) is used widely as a signalling molecule in biological systems including as a trigger for biofilm dispersal or detachment, e.g., by *P. aeruginosa* (Austin and Bergeron, 1995; Barraud et al., 2006; McDougald et al., 2012). NO in bacteria interacts with many bacterial regulatory components, such as OxyR, SoxR, NsrR, NorR and regulators of the *FNR* family (Spiro, 2007). Furthermore, NO has been shown to reduce platelet adhesion to extracorporeal circuits (Annich et al., 2000). In the case of *Nitrosomonas europaea*, the addition of > 30 ppm NO resulted in reduced biofilm formation and when applied below 5 ppm led to an increased numbers of motile and planktonic cells (Schmidt et al., 2004).

The delivery of exogenous NO is achieved by using NO-releasing compounds called NO donors. Examples of NO donors that have been investigated for use in biofilm dispersal include: sodium nitroprusside (SNP), S-nitroso-N-acetylpenicillamine; and S-nitroso-L-glutathione, 6-(2-hydroxy-1-methyl-2-nitrosohydrazino N-methyl-1-hexanamine and 1-(hydroxy-NNO-azoxy)-L-proline (Barraud et al., 2006; Barraud et al., 2009; Barnes et al., 2013). Small molecule NO donors possess broad-spectrum antibacterial properties against both Gram-positive and Gram-negative bacteria (Raulli et al., 2002; Charville et al., 2008; Hetrick et al., 2009).

Although biofilm dispersal responds to nutrient availability, oxygen fluctuations

and increase of toxic products, or other stress-inducing conditions as well as various carbon and nitrogen sources (Sauer et al., 2004; Karatan and Watnick, 2009; Rowe et al., 2010), the molecular mechanism underlying the dispersal response by NO involvement needs further study (McDougald et al., 2012).

1.7.3 Predictive modelling

With knowledge of the influence of temperature and time on thermophile growth, sporulation and detachment, it may be possible to better understand and manage spore-forming thermophiles in dairy powder plants. In this regard, the use of predictive modelling may be useful to extend the run time of dairy plants.

Predictive microbiology is used to quantify the microbial ecology of bacteria in foods, or other environments, by systematically studying microorganism behaviours, i.e. rate of survival/ inactivation, limits to growth (growth/no growth), and growth rates of specific organisms as influenced by environmental conditions (McMeekin and Ross, 1996; McMeekin and Ross, 2002). This means studying and quantifying various parameters such as lag time, generation time, maximum growth rate, and maximum cell concentration of microorganisms under particular conditions (Ross and McMeekin, 1994; Dalgaard et al., 1994; Tienungoon et al., 2000). Moreover, the models and studies are usually intended to contribute to Hazard Analysis and Critical Control Points (HACCP) and risk assessment (McMeekin and Ross, 2002). For example, a model was developed for the risk of listeriosis starting at

manufacturing but including all influences until consumption, i.e., ‘at the table’ (Tenenhaus-Aziza et al., 2014), and also for pathogenic microorganisms in raw milk and soft cheeses (Valero et al., 2014; Perrin et al., 2015).

Mtimet et al., (2015) studied *G. stearothermophilus* ATCC 12980 within the temperature range from 35°C to 72°C and various pH, generated growth rate data using a mathematical modelling which previously developed by Baril et al., 2012. They studied the sporulation ability of *G. stearothermophilus* ATCC 12980 in different conditions after a heat treatment. By using the collected data, the potential application of quantitative microbiological risk assessment techniques was demonstrated by running Monte Carlo simulation, Bayesian inference and sensitivity analysis (Pujol et al., 2013).

Comprehensive reviews of biofilm formation are found in Bridier et al. (2013; 2015) in which the authors observed that biofilms provided protection to cells exposed to stressful environmental conditions and that it requires innovative methods and techniques to overcome the problem of biolfilm formation. They used microscopic visualisation techniques to analyse the three-dimensional spatial arrangement of cells and their surrounding matrix in *Bacillus subtilis* biofilms in order to better understand biofilm architecture.

The Institut National de la Recherche Agronomique (INRA), France has been conducting research to reduce the need for harsh cleaning processes in food processing plants by modifying stainless steel surfaces to retard biofilm development. These surface modifications involve changing the micro-topography or nano-topography of the surface (Faille et al., 2014;

Cunault et al., 2015) to retard attachment.

The mathematical models used in predictive microbiology can be grouped into:

(a) Primary models which are used to describe the change in the number of target microbes over time, under specified conditions. To develop primary models, the Baranyi, modified Gompertz, and Logistic model equations can be applied to the observed data (Lee et al., 2014).

(b) Secondary models which are used to describe the effect of conditions (pH, a_w, temperature, atmosphere, or composition) studied, on the parameters of the primary model. A study by Ng and Schaffner (1997) characterised and modelled germination, outgrowth, and lag time and the exponential growth rate of *B. stearothermophilus* in salty carrot medium as a function of pH, temperature, and NaCl concentration.

(c) Tertiary models are ‘user-interfaces’ that use computer software programs to analyse collected data using mathematical formulae to produce graphs and predictions. A review of available predictive microbiology software tools (Tenenhaus-Aziza and Ellouze, 2015) presents an overall description and comparison of 16 software tools currently in use in predictive modelling. The software available includes databases, predictors, fitting tools, risk assessment tools, and the comparison also considered the environmental factors (temperature, pH, a_w, etc.), the type of media (broth or food) and the number and type of the microorganisms (pathogens and spoilers) included in the software tools.

Predictive models may not be able to provide exact predictions in all cases in food microbiology as there are many aspects that can affect changes in both food and its microbial ecology that are not included in all models. Therefore the application of predictive microbiology by microbiologists requires and understanding of the overall microbiology of the product (McMeekin et al., 2008).

1.7.4 Temperature shifts on biofilm development

When *G. stearothermophilus* attach to stainless steel in milk powder processing plants, they form biofilms that allow these microorganisms to multiply, sporulate and, eventually, be re-released into the product and to contaminate milk powders. The biofilm can protect the cell from harsh environments, including chemicals applied during CIP by preventing cells coming into direct contact with those chemicals. Biofilm formation can also disrupt flow rates, and may occur on parts of the plants that are hard to clean. Preventing or delaying biofilm formation may be a more effective means to extend run times than improved cleaning.

As described above, there have been numerous approaches to control or eliminate biofilms of thermophiles in milk processes including cleaning procedures and equipment re-design, but the problem remains throughout the world. As noted above, *G. stearothermophilus* can grow at temperatures from ~45 to 70 °C in production lines of dairy plants. Within this growth range, thermophilic bacilli show different responses especially during temperature shifts. In the range 45 to 65 °C, Hasegawa et al. (1980) reported changes in

lipids and fatty acid composition. The activity and stability of several enzymes was also affected (Lauwers and Heinen, 1983), including synthesis of new proteins (Wu and Welker, 1991; Weber and Marahiel, 2003; Tjalsma et al., 2004) as well as nutritional requirements (Souza and Martins, 2001). The different responses to temperature may be able to be exploited to better control thermophile growth in processing lines.

Knight et al. (2004) found a method to delay biofilm build up using temperature cycling to interrupt the growth cycle of *Streptococcus thermophilus* and reduced their growth at their optimum condition. These responses to changing environmental conditions, might play a role in the early stages of biofilm formation (Van Wamel et al., 2007).

1.8 Thesis objectives

The objective of this work was to investigate the kinetics of thermophilic spore-former growth, biofilm development and spore formation as a function of temperature and water activity, and to develop an understanding of how these factors influence thermophile attachment and biofilm formation on stainless steel and affect the time before spore release into milk during commercial milk powder processing.

The following objectives are addressed in this thesis:

1. To characterise the growth rates of strains of *Geobacillus* spp. isolated from Australian milk powder plants, over their full temperature

range for growth using various broth model systems. The data, including variability, were described using a square root model (Ratkowsky et al., 1983).

2. To observe the time-course of attachment and proliferation of *G. stearothermophilus* cells and spores using a bench flow-through laboratory reactor and to test the effect of different temperatures and flow rates on the time to spore formation and release. Experiments were done at temperatures from 45 to 67.5 °C, relevant to pasteurisers and evaporators in milk powder plants, by immersing the reactor in a temperature-controlled water bath.
3. To observe the time-course of attachment and proliferation of *G. stearothermophilus* W14 cells and spores using a bench-scale flow-through laboratory reactor and to test the effect of different temperatures cycling and flow rates on spore formation.
4. To explore the potential of nitric oxide to induce detachment of *G. stearothermophilus* biofilms before production of high levels of spores to enable longer run times in dairy powder processing.

2. Modelling Growth Rates of *Geobacillus* spp. isolated from Australian Dairy Milk Powder Plants as a Function of Temperature

2.1 Introduction

As discussed in Chapter 1, *Geobacillus stearothermophilus* and related species are Gram-positive, thermophilic, spore-forming bacteria often found as the main spore-forming contaminants in milk powder plants (Molva et al., 2009). They are frequently found in warm sections of milk processing lines and in end products (Flint et al., 1997; Scott et al., 2007). The main contaminant species is *G. stearothermophilus*. Like the similarly problematic *Anoxybacillus* spp., it is a non-pathogenic facultatively anaerobic bacterium forming highly heat-resistant endospores and biofilms originating from vegetative cells or spores attached to stainless steel equipment or other food contact surfaces (Chen and Hotchkiss, 1993; Barnes et al., 1999; Aureli et al., 2011). Adhesion to surfaces is aided by the hydrophobic properties of the cell and spore walls (Faille et al., 2002).

The types and levels of thermophilic spore-forming bacteria found in raw milk depend on several factors; i) milk collection season, ii) milk composition, iii) sanitary conditions during raw milk handling and transport, and iv) treatment conditions throughout milk processing. Specific operational procedures used in individual factories and dairies can influence specific strains present in heat-treated end products (Vissers and Driehuis, 2009). Additionally, biofilms can arise from residual spores remaining after CIP processes.

During milk powder production, proliferation of thermophilic bacilli is selected due to heat treatments, especially during pasteurisation and evaporation, where temperatures range from 40 to 80 °C (Verdurmen and de Jong, 2003). Temperatures in the range 40 to 75 °C are generally considered to permit growth of thermophilic bacilli (Burgess et al., 2010). Thermophilic spore-forming bacteria may survive dairy powder processing and, within the growth permissive temperature range, establish biofilms, especially after heating phases.

A mathematical model estimating the growth rate of contaminating spore-forming bacteria is potentially useful to extend dairy plant run times if spore counts can be predicted in powdered milk based on temperature and time. Equally, it could be useful to identify sites in powder processing plants where biofilms would form most rapidly, and where additional controls might be applied to extend run times. Predictive models have been developed to rapidly estimate bacterial populations in, or on, food based on physico-chemical criteria and time, especially in reducing the pathogen risk to humans (Dalgaard et al., 1994; Baranyi and Roberts, 1995; Mellefont et al., 2003a.b; Membré and Lambert, 2008). Until recently there have been no growth models for *Geobacillus* spp. in the published literature or in databases such as ComBase (www.combase.cc). Mtimet et al. (2015), however, presented a model for the growth of *G. stearothermophilus* ATCC12980, the type strain originally isolated from spoiled canned vegetables, in response to temperature and pH.

In this Chapter experiments to generate growth rate estimates of a variety of strains of *G. stearothermophilus* as a function of temperature, and the consequent results, are presented. The experiments conducted to generate the growth rate data sought are summarised in Appendices 1 and 2.

Various methods were used because of the difficulties in working with this microorganism. From this work, the results of Mtimet et al. (2015) are reinforced and extended using multiple strains of *Geobacillus* spp., mostly *G. stearothermophilus*. Specifically, this involved determining and analysing growth rates of 16 strains of *Geobacillus* spp., isolated from Australian milk powder plants, over a temperature range from 45 to 70 °C, using various broth model systems. The effect of water activity was also explored using a single, representative strain as was the effect of aerobic and anaerobic growth rates and media composition. The data, including variability, are described using a four-parameter square root model (Ratkowsky et al., 1983).

Despite erratic growth rate responses the predictive growth model derived for *Geobacillus* spp. offers a means to estimate the effects of temperatures and water activity on growth rate of vegetative cells of *Geobacillus* spp. and, for milk powder processing operations, offers the possibility to identify regions where growth and biofilm formation are most likely to occur and to occur most rapidly. This model will assist in definition of the points in milk powder processing plants at which *Geobacillus* spp. can grow most rapidly, taking into account strain and species diversity and variation.

2.2 Materials and Methods

2.2.1 Bacterial strains: Identification and inoculum preparation

Sixteen presumptive *Geobacillus* spp. (strains 103A-, W5A, W5B, W5D, 3D, 50B, 28S, T1C, W7E, 126, 291, T80, W16, W7D, T17, and W14), originally isolated from Australian milk processing plants, were generously provided by Drs. C. Pillidge and I. Powell of Dairy Innovation Australia, Ltd. DNA was extracted using UltraClean™ DNA isolation kit (MO BIO Laboratories, Inc., Carlsbad, California, United States): DNA extraction was performed as described by the manufacturer. Species-level identification was undertaken using PCR amplification of the 16S rRNA gene fragment with universal primers 10F (5′-AGT TTG ATC ATG GCT CAG ATT G-3′) and 907R (5′-CCG TCA ATT CCT TTR AGT TT-3′).

The isolates were identified based on their 16S rRNA gene sequences. PCR was performed as follows: all PCR reactions contained 1× Standard PCR Buffer, 4 μL of 10 mM dNTPs, 10 pmol/μL of each primer, and 5 U/μL of *Taq* DNA polymerase in 100 μL (MO BIO Laboratories, Inc., Carlsbad, California, United States). Amplification was performed in a PTC-200 programmable peltier thermal cycler (MJ Research, Inc., Watertown, Mass., USA) with 1 cycle of 94 °C for 1 min followed by 32 cycles of denaturation (60 s at 94 °C), annealing (60 s at 57 °C), and extension (60 s at 72 °C), with a final extension of 72 °C for 10 min. For analysis, 10 μL of reaction mixture was electrophoresed on a 1% agarose gel and stained with ethidium bromide

solution (5 µg/L). Purified PCR products were sequenced (Macrogen Inc., Seoul, South Korea, http://www.macrogen.com), and similarity searches were performed using the National Centre for Biotechnological Information online program BlastN (Altschul et al., 1990) against nucleotide sequences stored in the GenBank database. The sequences were edited with BioEdit software (Hall, 1999).

Stock cultures were maintained at −80 °C in 15% glycerol suspensions in Brain Heart Infusion broth (BHIB, Oxoid CM0225). Prior to the commencement of each experiment, the isolate was resuscitated from storage by plating onto Tryptone Soya Agar (TSA, Oxoid CM0129 with 15 g/L grade J3 Agar A120218, Gelita, Australia Pty Ltd) and incubated at 55 °C for 24 h.

2.2.2 Exponential phase inocula

An inoculum from a single colony from a 24 h plate culture was added to 30 mL BHIB in a sterile 50 mL centrifuge tube and incubated with shaking (150 rpm; New Brunswick Innova 44 Incubator Shaker) at 55 °C for 10 h. 100 µL of that culture was transferred to 30 mL BHIB and incubated for a further 10 h at 55 °C, to prepare an exponentially growing culture. Exponential phase inocula were added to a variety of growth media and growth monitored at a range of temperatures as described in Section 2.2.6, below.

2.2.3 Spore suspensions

Exponential phase inocula were divided into 5 mL aliquots in six sterile 15 mL centrifuge tubes and boiled for 30 min at 100 °C to kill vegetative cells. The suspensions were cooled to room temperature and then 1 mL transferred to 1.5 mL sterile micro centrifuge tubes. The spores were collected by centrifugation at 3,486 × *g* (Eppendorf centrifuge 5417R, B. Braun, Singapore) at 25 °C for 10 min. The supernatant was decanted and the pellets (containing the spores) in each tube were re-suspended in 1 mL of reconstituted sterile skim milk (see Section 2.2.4), or BHIB, as appropriate.

2.2.4 Growth media

A combination of commercially available media and broth preparations were used in this study.

BHIB was prepared as per manufacturer's instructions and stored at 4 °C prior to use. This medium was used for turbidimetric studies at all temperatures.

Reconstituted Skim Milk (RSM) was prepared by dissolving 100 g of powdered skim milk purchased at retail (Woolworth's Home Brand) in 1 L distilled water, and autoclaving at 110 °C, 5 psi for 5 min. The short autoclave time was required to minimise caramelisation of the medium. Prior to each experiment the presence of thermotolerant microorganisms in the RSM was assessed by plating 100 µL onto the surface of a TSA plate and incubating at

55 °C for 24 h. Due to its opacity, RSM was not suitable for turbidimetric growth determinations because of its inherent turbidity as a milk-based medium and was used only in bioreactor experiments when cell/spore concentrations could be determined by viable count.

2.2.5 Water activity modified milk

RSM was prepared as described in (Section 2.2.4). Additionally, RSM at different a_w was prepared by dissolving different amounts of the milk powder into 100 mL distilled water, thereby creating different milk/water ratios (w/v) as follows: 1:10 (10 g: 100 mL), 1:6.6 (15 g: 100 mL), 1:5 (20 g: 100 mL), and 1:4 (25 g: 100 mL). Water activity of the concentrated reconstituted skim milk was determined from triplicate measurements of sterilised and cooled RSM using an Aqualab CX2 water activity meter (Decagon Devices Inc., Pullman, USA). The pH of each reconstituted milk preparation was determined before inoculation. The reported values were calculated by taking the average of all batches of milk prepared at the various concentrations.

G. stearothermophilus (strain W14) was used in these experiments because this strain displayed more reproducible growth responses than other strains (see Figure 2.1) but was also representative of the growth rate responses of most other strains.

2.2.6 Construction and analysis of growth curves

Growth rate data were derived by various methods including viable counts and turbidimetry. Log_{10} (OD_{600}) or Log_{10}CFU were plotted against time and

growth curves analysed as described below. In some experiments using the Bioscreen differences in detection times of serial dilutions of a culture were used to estimate generation times and, from that, growth rates as described by Biesta-Peters et al. (2010).

2.2.6.1 Bioscreen

Detection Time Method

A Bioscreen C turbidimeter/growth analyser (Oy Growth Curves AB Ltd., Helsinki, Finland) was used for most growth rate assays. Five serial doubling dilutions of cultures of *Geobacillus* spp. were inoculated into wells of a Bioscreen 200-well Honeycomb plate (20 µL of an appropriate dilution of an exponential phase inoculum into 280 µL of BHIB in each well). In a separate set of experiments ten-fold dilution was used, with five replicates of 30 µl of exponential phase dilutions inoculated into 270 µL of BHIB. Generation times were estimated as the average of the difference in detection times for doubling dilutions. Where 10-fold dilutions series were used the average of the difference in detection times divided by 0.301 ($\log_{10}2$) was taken as the generation time. All well plates were incubated with “moderate” shaking applied for 20 seconds before measurements were taken by the instrument.

Growth Curve Method

Absorbance readings at 600 nm (wideband range) were taken every 20 min for 24 to 120 h depending on the temperature of incubation. The Bioscreen instrument does not allow the optical density (OD) scale to be calibrated to the OD of the basal medium that, in many experiments, was high in comparison

to the final OD readings obtained. Accordingly, OD readings were corrected by making the background OD value equivalent to 100% transmittance, and calculating the transmittance of the observation on this corrected scale. The corrected transmittance value was converted to an equivalent OD. These calculations were done using Microsoft® Excel. Growth rates were then calculated from these recalibrated OD values, using the methods described in Section 2.2.6.4. Bioscreen OD growth curves in BHIB were generated for various strains at various temperatures <59 °C.

2.2.6.2 Shaking flask cultures

At temperatures higher than 59 °C, OD determinations in BHIB culture were not possible due to darkening of the media over time of incubation and because of the operating limits of the Bioscreen C instrument. Accordingly, to generate data at higher temperatures, 50 mL of pre-warmed BHIB was added to sterile 125 mL conical flasks. The broths were temperature equilibrated in a shaking water bath (Ratek Instruments Pty. Ltd., Boronia, Australia) at 60, 61, 64, 65, 66, 67, 68, 70, 75 °C prior to inoculation and incubated with shaking (80 oscillations/min). One mL of exponential phase inoculum was added to 50 mL of media. To monitor growth, a 500 µL aliquot was removed at hourly intervals and serially diluted in 0.1% Bacteriological Peptone Water (Oxoid LP0037). 100 µL aliquots of appropriate dilutions were then surface-plated on TSA and incubated for 24 h at 55 °C before counting colonies to determine viable counts.

2.2.6.3 Bioreactors

One litre working volume bioreactors (BioFlo/CelliGen 115 bench top bioreactor, New Brunswick Scientific, Edison, New Jersey, USA), including inbuilt pH and oxygen probes, were used to generate growth rate data under aerobic and anaerobic growth conditions. For these experiments 300 mL of cooled RSM was added to bioreactors at 55, 60, 65 or 70 °C with agitation set at 150 rpm, prior to commencement of each experiment. For aerobic conditions, the oxygen level was set to 20%. Anaerobic conditions were achieved by sparging the RSM in the vessel with 100% nitrogen prior to the commencement of each experiment. The pH probe was calibrated at pH 7 and 4 by connecting it to the control/utility station of the bioreactor (before autoclaving the vessel and the media). The dissolved oxygen probe was set at 100% dissolved O_2 after autoclaving, by connecting to the control/utility station of the bioreactor, and left for a minimum of 8 h. After 8 h, the dissolved O_2 probe was calibrated to 0% by sparging with 100% Nitrogen gas for 20 min.

G. stearothermophilus W14 was used for all bioreactor experiments. Once the RSM had reached the desired temperature, samples were aseptically withdrawn from the vessel for viable count determinations: i) prior to inoculating with 0.6 mL of spore suspension (see Section 2.2.3) through the injection port, ii) immediately after injection, and iii) hourly, thereafter. Viable counts were monitored until the pH decreased to pH 4 (approximately 15 to 18 h for each experiment). Plates were incubated for 24 h at 55 °C before colony enumeration. Spore counts in each sample were enumerated by

boiling 1 mL of sample at 100 °C for 30 min, followed by surface-plating of 100 µL aliquots of appropriate dilutions on TSA. Plates were incubated for 24 h at 55 °C before colony enumeration to determine spore counts.

2.2.6.4 Analysis of growth curves

Data generated using the various growth media and methods described above yielded growth curves of varying quality and utility. Not all strains exhibited growth in all media at all temperatures and in some cases the maximum population density observed by turbidimetry was also affected. When using turbidimetric techniques it is usual to set the scale of the optical density measurements to 0 using un-inoculated media. This adjustment is not possible using the Bioscreen instrument. Accordingly all Bioscreen OD measurements were rescaled as described above before growth rate calculation to account for the initial optical density of the medium.

Further, in many cases the data generated were not able to be fitted using sigmoid curve fitting software, and kinetic parameters were estimated using linear regression through the steepest part of the growth curve. Using this simplified approach, it was necessary to determine objective estimates of the steepest tangent to the curve. To do so, tangents were calculated systematically on sub-sets of sequential observations, e.g., the slope of the line of best fit through observations 1 to 10, the slope of the line of best fit through observations 2 to 11, and so on for all data. After experimentation it was concluded that 13 sequential observations provided the most consistent growth rate estimates for this process, and the steepest slope from any set of

13 successive observations was taken as the relative growth rate estimate. Nonetheless, even after this process, it was necessary to define criteria for inclusion of data in the kinetic parameter analysis.

OD data were only included in analyses if the growth curve exhibited ≥ 5 data points measured in the exponential phase of growth. Data that displayed overtly biphasic or triphasic growth responses were excluded from analyses. Generation time (h) was calculated by dividing the slope of the regression line, '*m*', by 0.301 (equivalent to $\log_{10}2$). The complete data set of strain, growth medium, culture system, temperature, enumeration method and generation time estimates are presented in Appendix 1.

The Ratkowsky et al. (1983) model;

$$\sqrt{rate\ (generations.h^{-1})} = b \times (temperature - T_{min}) \times \left\{1 - exp^{[c \times (temperature - T_{max})]}\right\}$$

Equation 1

where b is a regression coefficient, T is the temperature (°K), T_{min} is the notional minimum temperature for growth obtained by solving for the lower temperature for which the rate predicted from the regression is zero, *c* is a regression coefficient, and T_{max} is the notional maximum growth temperature (°K) obtained by solving for the upper temperature for which the rate predicted from the regression is zero, was fitted to all growth rate datasets for individual species or strains as (√ 1/generation time) *vs*. temperature using the

nonlinear regression programme SAS PROC NLIN. The datasets for all growth rates for individual species or strains is presented in Appendix 1.

The model was fitted to the growth rate data. Due to the variability in the data sets for each individual strain/species, the data for all strains and species combined was also modelled using the Ratkowsky et al. (1983) model, with modifications as described below.

McMeekin et al. (1987) introduced a model to describe the combined effect of temperature and water activity on the growth rate of bacteria, and other microbes. The term describing the effect of water activity in that model was:

$$\sqrt{rate} = \ b' \times (\sqrt{(a_w - a_{w\,min})}$$

Equation 2

where b' is a constant to be fitted, a_w is the measured water activity in the growth environment, and a_{wmin} is a parameter to be estimated from that data that represents the theoretical lowest water activity at which growth can occur, and is derived from the value of the fitted regression line when the predicted rate = 0. This term has been used in numerous published models.

Because only one data set was available for evaluation of the effects of water activity, the water activity term in the model was based on a cardinal parameter model term to express the relative effect of a_w as a function of the optimal water activity for growth, the actual a_w of the environment, and the

derived value of a_{wmin}. The term used is based on the term in Equation 2, but rearranged, as per Mejlholm and Dalgaard (2009) into a dimensionless expression that expresses the relative effect of sub-optimal water activity on growth rate:

$$relative\ growth\ rate\ inhibition = \frac{(a_w - a_{w\,min})}{(a_{w\,opt} - a_{w\,min})}$$

Equation 3

where a_w and a_{wmin} have the same meanings as given above, and a_{wopt} is the water activity at which the growth rate is optimal. For the purposes of the modelling, it is assumed that a_{wopt} is 0.997, which is a typical value for non-halophiles.

2.3 Results and discussion

Thirteen isolates were identified as *Geobacillus stearothermophilus*, with the remaining strains being identified as *Geobacillus kaustophilus* (strain 3D) or *Geobacillus thermoleovorans* (strains 50B and T80).

Collectively, the data gathered from Bioscreen C, waterbaths and bioreactors yielded >300 data sets. However when the data were filtered using the criteria described in Section 2.2.6.4, only 201 of the data sets could be used for fitting to the square root model (Ratkowsky et al., 1983). Further, preliminary studies using a range of growth media revealed growth rates varied widely according to the composition of the culture medium.

Among the media tested *Geobacillus* spp*.* grew most consistently in BHIB, possibly because it contains 0.2% glucose rather than lactose which most *G. stearothermophilus* strains apparently do not metabolise (Goodman and Pederson, 1976). Nonetheless, anecdotal reports indicate that many dairy strains of *G. stearothermophilus have* acquired the genes for lactose fermentation.

Even for a single medium and strain combination, variability in growth rates occurred and was much greater than experienced in previous studies with psychrotrophic and mesophilic vegetative bacteria (Meer et al., 1991, Mellefont et al., 2003a.b; Buehner et al., 2015), and is also reflected in the experience of Mtimet et al. (2015), who studied *G. stearothermophilus* ATCC 12980 at various temperatures and pH. Additionally, the type of outgrowth media determined the method of data capture, because opaque or temperature sensitive media necessitated the use of viable counts whereas translucent media could be monitored turbidimetrically for most incubation temperatures.

2.3.1 Growth rates in various media

Growth media used in this study included BHIB and RSM. Growth curves for 16 strains of *Geobacillus* spp. (each studied at 5 to 10 different temperatures in the range 45 – 70 °C) were generated (*see* Table 2.1). The number of datasets considered to be acceptable for the model fitting process are summarised in Table 2.1.

Growth rates of *G. stearothermophilus*, *G. kaustophilus* and *G. thermoleovorans* varied with temperatures or media used (Appendix 1). In addition to using BHIB and RSM as growth media, other media, including soluble starch, D(+) glucose, casein, lactose, casamino acids, yeast extract, $CaCl_2$, and bovine serum albumin, were employed in preliminary studies to determine growth potential as assessed by lag time and generation time. However, utilising media other than BHIB and RSM produced very slow growth for *Geobacillus* spp; therefore those data were eliminated from the data set used to construct the model. BHIB was more effective in growing *Geobacillus* spp. reproducibly over a wide range of temperature (45.5 to 59 °C). The minimum observed growth temperature for *Geobacillus* spp. was 45.5 °C, consistent with reports of a lower temperature limit for growth of 35 to 47 °C (Burgess et al., 2010). Moreover a study of *G. stearothermophilus* ATCC 12980 growth over the temperature range from 41.5 to 65 °C estimated the cardinal temperatures T_{min}, T_{opt} and T_{max} were 38.5, 57.6 and 68.0 °C (Mtimet et al., 2015) respectively. This analysis indicated apparent fastest generation times of 9 to 12 min at ~60 °C. Generation times in the range 12 to 29 min, found when using strain W14, are similar to those reported by Flint et al. (2001) using a flow-through bioreactor, operated in either circulated or uncirculated modes, at 55 °C where minimum generation times of 19 to 25 min were reported.

Growth rate data for *G. stearothermophilus* strain W14 under either anaerobic (100% N_2) or aerobic (20% O_2) conditions were generated using the bioreactor at 55, 60, 65, and 70 °C (Figure 2.2).

Table 2.1. Number of growth curves generated and fitted within the criteria for strains of *Geobacillus* spp., indicating temperature and methodology used; highlighted sections refer to the turbidimetric data.

		Number (n) of Accepted Generation Time Estimates																		
Media	Methods	Temperature (°C)																		
		45	45.5	47.5	49	50.5	51	52.5	53	55	55.5	57.5	58	58.5	59	60	61	63	65	70
BHI	Bioscreen C (Calibrated OD)	13	13	14		14		14			14	14		13	14					
	Bioscreen C (Doubling dilution)	1			1		2		12	8			9		13					
	Waterbath (OD)															3			3	3
	Waterbath TVC															3	2	3	4	3
RSM	Bioreactor aerobic									1						1			1	1
	Bioreactor anerobic									1						1			1	1
Growth curves (n)		14	13	14	1	14	2	14	12	10	14	14	9	13	27	8	2	3	9	8

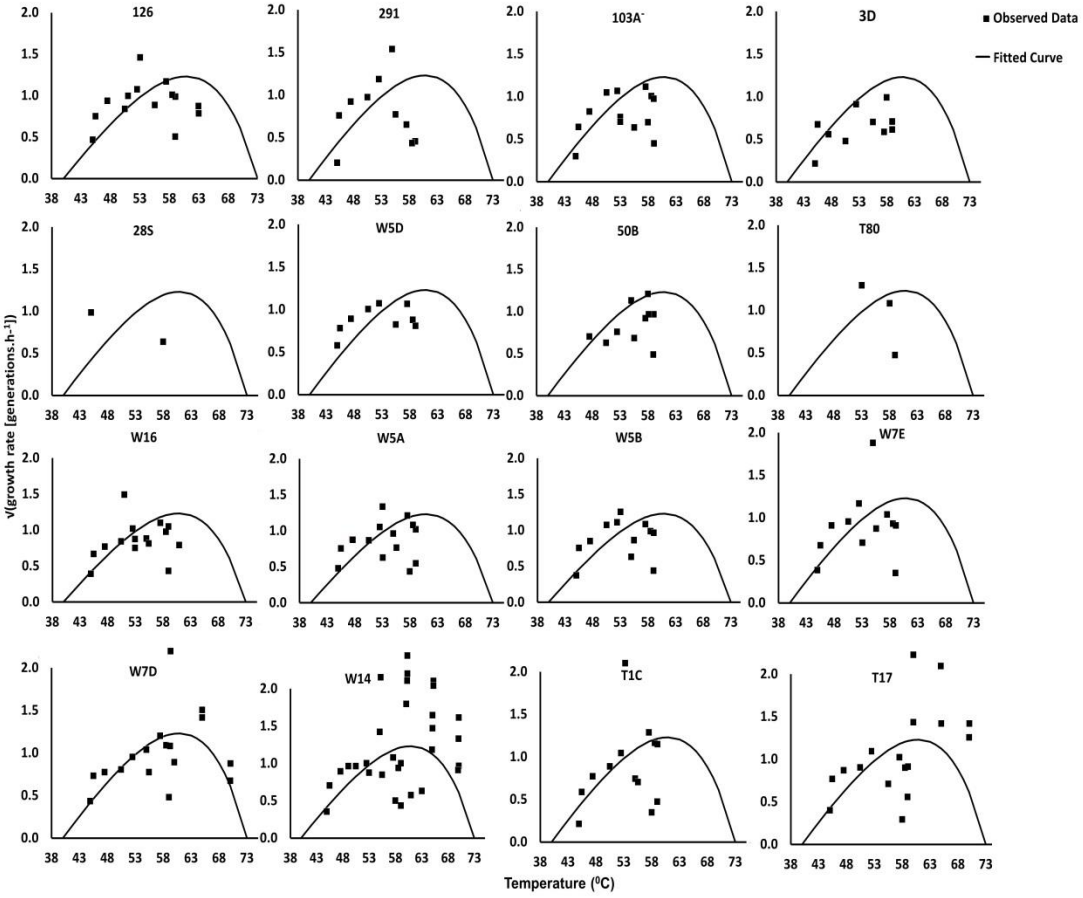


Figure 2. 1 Effect of temperature on growth rates of *Geobacillus* spp. in BHIB and RSM, fitted with a secondary model (Equation 1) to all data simultaneously and shown in comparison to individual strain datasets.

G. stearothermophilus strain W14 showed fastest growth at 60 and 55 °C in aerobic and anaerobic conditions respectively with generation times of 12 to 13 min (Appendix 1). The slowest growth was observed in anaerobic conditions at 55 and 70 °C with generation times of 29 to 34 minutes respectively.

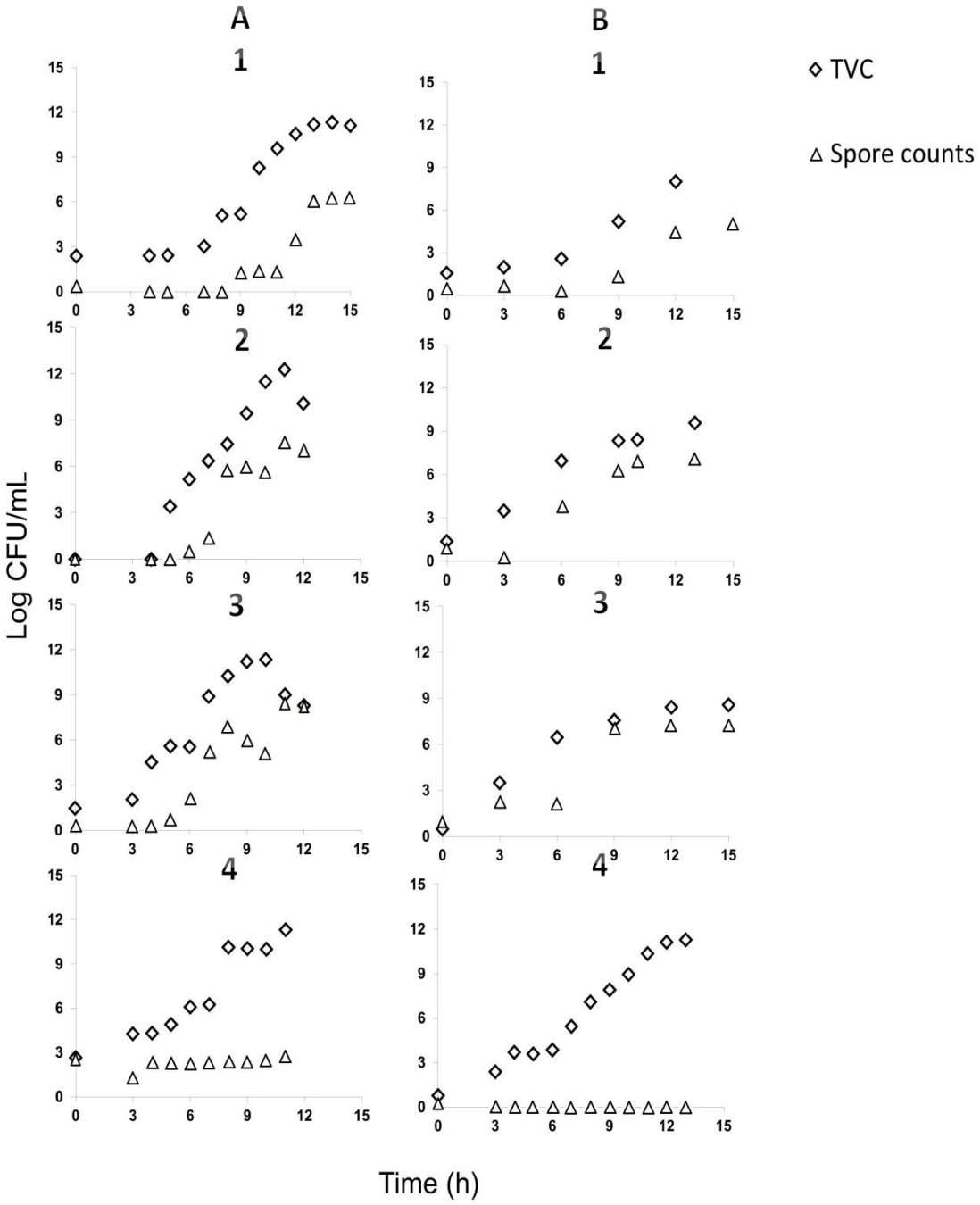


Figure 2. 2 Aerobic (A) and anaerobic (B) growth of *G. stearothermophilus* W14 vegetative cells (◇) and spores (Δ) in bioreactors at 1) 55 °C; 2) 60 °C; 3) 65 °C; and 4) 70 °C in RSM media.

2.3.2 Water activity and growth rate

The a_w of concentrated RSM (Table 2.2) suggests that during the production of milk powder the a_w of the milk decreases from approximately 0.992 (milk) to 0.265 (milk powder). It is evident from the a_w values presented in Table 2.2 that reducing the ratio of milk powder to water did not achieve water activities that were evenly distributed in the range between milk and milk powder, even though for some preparations more than double the amount of milk powder recommended by the manufacturers was added.

Table 2. 2 Water activity and pH measurements for different milk powder *vs.* water ratios

Milk powder (g)	Water (mL)	Ratio (skim milk: water)	a_w	pH
10	100	1:10	0.992	6.59
15	100	1:6.6	0.991	6.45
20	100	1:5	0.990	6.34
25	100	1:4	0.988	6.30

Additionally Table 2.2 shows the measured pH of the various concentrated reconstituted skim milk powders. These values were calculated by taking the average of all batches of milk prepared at the individual ratios. A decrease in the pH of the concentrated milks from 6.59 to 6.30 corresponds to an increase in the concentration from ‘normal’ (10 g per 100 mL water) 2.5 x concentrated milk. These levels, however, are not expected to inhibit *Geobacillus* growth Burgess et al. (2010) reported growth over the pH range 6.0 to 8.0. Mtimet et al. (2015) reported more detailed growth rate data that show that growth rate

at pH 6.6 might be in the order of 5% faster than at pH 6.3.

The evaporator and spray dryer processes are the points at which the water activity of the milk/milk powder is most greatly reduced to levels that make the product shelf stable. A water activity of 0.86 will not support growth of most bacteria of interest in foods (Labuza, 1980) and will not support growth of *G. stearothermophilus* based on the results in Figure 2.5, which suggests that the lower a_w limit for growth of *G. stearothermophilus* of ~0.95.

Figure 2.3 shows the growth curves of *G. stearothermophilus* W14 at 60 °C for various combinations of reconstituted skim milk. While large differences in growth rate as a function of milk concentration/water activity are not apparent at this temperature, at other temperatures the effect of water activity on growth rate was more pronounced (data not shown).

Data from four temperatures were available but growth was not recorded under all conditions. In some cases, growth was erratic and rates could not be determined from the data obtained. In other cases, the temperature seemed to preclude growth during the duration of the experiment. Table 2.3 shows the estimated generation time (h) of *G. stearothermophilus* strain W14 at temperatures from 55 to 70 °C in various concentrations of RSM.

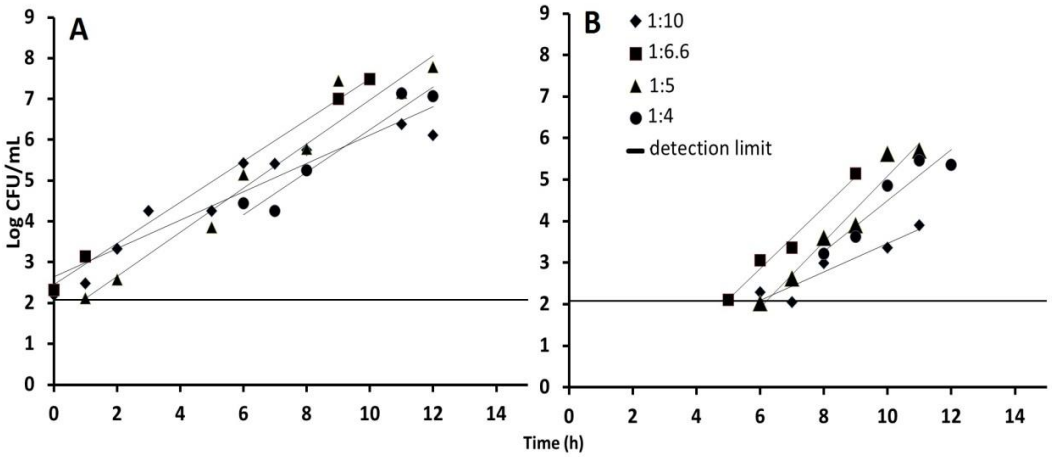


Figure 2. 3 Growth of vegetative cells (A) and spores (B) of *G. stearothermophilus* W14 at 60 °C in various concentration of reconstituted skim milk, showing the relatively minor effect under some conditions, but also the extent of variability.

The data were graphed and, where the data permitted, linear regression was applied to the data to generate estimates of a_{wmin}, as shown in Figure 2.4. While data for 60 °C are shown, they were not used to estimate a_{wmin} because the data are such that no realistic growth rate estimate was possible. From the data sets for 70 °C, 65 °C and 55 °C three estimates of a_{wmin} were generated. As with other studies associated with this microorganism, the results were relatively erratic and potential outliers were identified in the 65 °C and 70 °C datasets. For interest, these outliers were removed and the a_{wmin} values estimated again. From both sets of estimates it was concluded than an a_{wmin} of 0.983 was most representative of all the available data. Note also, that in these experiments there was consistent growth at 70 °C at a range of water activities.

Table 2. 3 Generation time (h) of *G. stearothermophilus* W14 in different

temperatures and water activity

Temperature (°C)	Ratio (skim milk: water)	a_w	Generation Time (h)
70	1:10	0.992	0.67
	1:6.6	0.991	0.89
	1:5	0.990	0.89
	1:4	0.988	0.94
65	1:10	0.992	1.06
	1:6.6	0.991	1.31
	1:5	0.990	4.58
	1:4	0.988	2.01
60	1:10	0.992	0.86
	1:6.6	0.991	0.60
	1:5	0.990	0.56
	1:4	0.988	0.60
55	1:10	0.992	nd
	1:6.6	0.991	nd
	1:5	0.990	0.54
	1:4	0.988	0.71
50	1:10	0.992	nd
	1:6.6	0.991	0.75
	1:5	0.990	nd
	1:4	0.988	nd

nd = growth was not detected

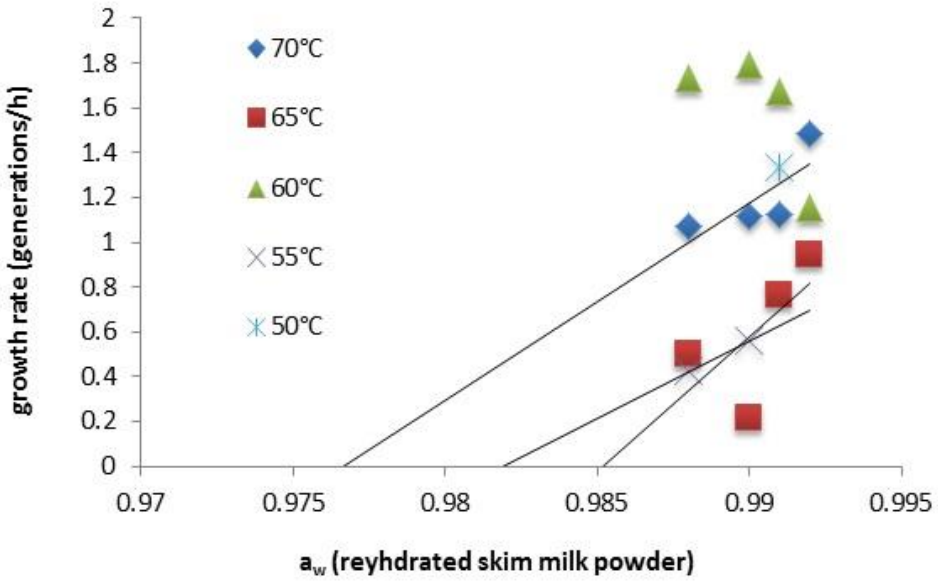


Figure 2. 4 Growth rate data as a function of water activity in reconstituted skim milk powder for *G. stearothermophilus* W14 at five different temperatures.

To translate the a_{wmin} information into a more practical value, relevant to milk powder processing, a_w values of milk were plotted against the water content of the reconstituted milk. The results are presented in Figure 2.5 and show that growth of *G. stearothermophilus* W14 would be expected to cease once the water content of the milk drops to below 30% of its original value.

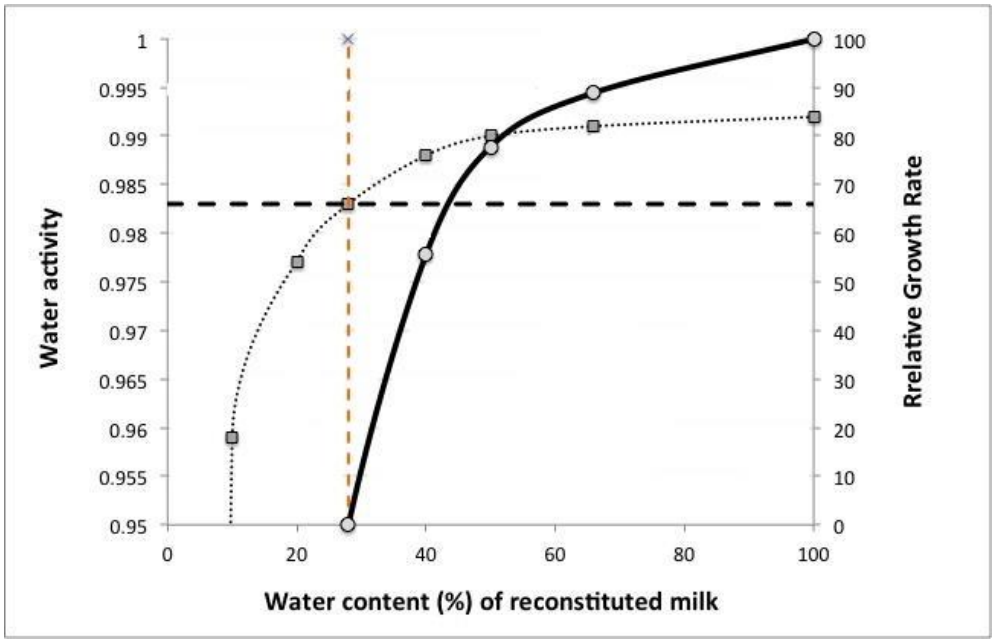


Figure 2. 5 Relationship between water activity (square symbols and dotted line) and moisture content (solid line and circle symbols) of rehydrated milk powder and showing the water activity level at which growth of *G. stearothermophilus* W14 would be expected to be completely inhibited. The solid black line shows the relative growth rate of *G. stearothermophilus* W14 as a function of water activity.

2.3.3 Mathematical model using growth rate data generated by different methods

Typically, when data for growth rates of bacteria are plotted against temperature, a consistent pattern of response is observed for a very wide range of species (Corkrey et al., 2012; Corkrey et al., 2014), and can be described well by the cardinal parameter-type models (Rosso et al., 1993), including the Ratkowsky et al. (1983) model. The typical response is characterised by a lower, theoretical, temperature termed T_{min} at which the

growth rate is predicted, by extrapolation of the fitted curve, to be zero. Above T_{min} the square root of growth rate increases in proportion to the temperature above T_{min}, until the rate of increase begins to slow, achieving a maximum growth rate at T_{opt}, (i.e., the temperature at which the growth rate is maximal). The square root transformation also contributes to the statistical rigour of the modelling process (Ratkowksy et al., 1991; Alber and Schaffner, 1992; Dalgaard et al., 1994; Ratkowsky et al., 1996). For this reason, such data are often presented as a plot of square root of growth rates *vs* temperature; i.e., a so-called 'square root plot' (Ratkowsky et al., 1983).

The square root of observed growth rate (√[generations/h]) *vs* temperature (°C) (*n*= 201, 16 strains, 4 enumeration/growth rate estimation methods) were fitted using the four-parameter square root model (Equation 1).

Generating growth rate data at temperatures above 70 °C presented technical difficulties, including that these data infer the T_{max} value is 73 °C, and because it is very difficult to generate growth rate data at temperatures near to the limits for growth. Partly for this reason and because of the relative paucity of data above 70 °C, the converged model without constraints on parameter values produced unrealistically low values of T_{max}. Accordingly, the model fitting was constrained to force T_{max} value is 73 °C. For similar reasons, the model was forced to converge to T_{min} value is 40 °C. The resulting fitted model is:

$$= 0.106 \times (temperature - 40) \times \left\{1 - exp^{[0.066 \times (temperature - 73)]}\right\} \mp 0.3483\ (RMSE)$$

Equation 4

Based on the a_{wmin} estimation process described above, the model was expanded to include a_w effects, thus:

$$\sqrt{rate\ (generations.h^{-1})} = 0.106 \times \left(\frac{(a_w - 0.983)}{(0.997 - 0.983)}\right) \times ((temperature - 40) \times \left\{1 - exp^{[0.066 \times (temperature - 73)]}\right\} \mp 0.3483\ (RMSE)$$

Equation 5

The results for the fit to the full, edited, data set are shown in Figure 2.6.

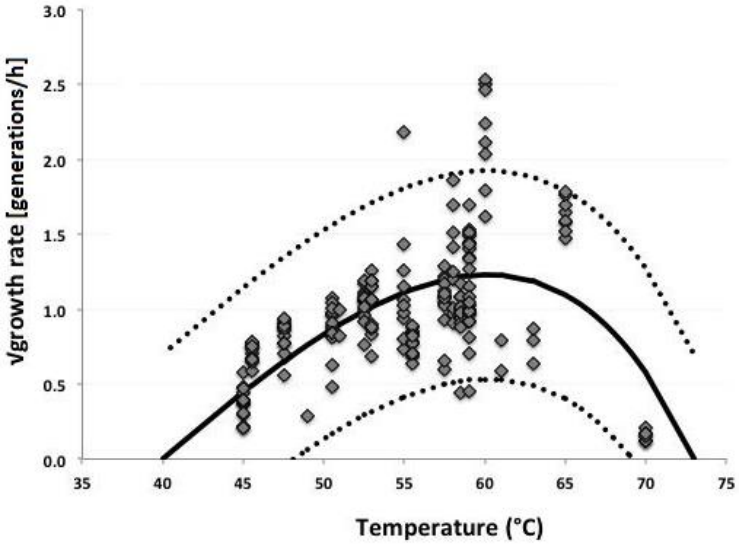


Figure 2. 6 Ratkowsky-type model fitted to the pooled data sets (see Appendix 1) and showing the 95% confidence interval on model predictions.

The T_{opt} values is ~60 °C, indicating that, as expected, all strains were

thermophilic. The proposed T_{max} value (73 °C) is unexpectedly high compared to the estimated optimum temperature for growth rate. It is generally observed that the temperature span between the optimum and maximum temperatures for growth rate for any microorganism is usually 5 to 10 °C (Ratkowsky et al., 2005).

The growth data for the 16 individual species/strains (Figure 2.6) showed a reasonable fit to the global model (Equation 4) to data obtained using different methods, strains and equipment, but the unexplained variability in the growth rate data is large compared to many other datasets for other species of vegetative bacteria in our experience and presented in the published literature (Flint et al., 2001; Corkrey et al., 2014; Mtimet et al., 2015).

Nonetheless, Equation 5 provides a useful description of the temperature dependence of *G. stearothermophilus* growth rates and limits. The estimated T_{min} value is 40 °C, although growth was not observed for any strain at temperatures below 45 °C. As discussed below, other workers have reported growth at temperatures in the range 40 to 45 °C also.

2.3.4 Growth rate estimates from turbidimetry methods (OD) and viable counts

The growth rate data were generated from various methods, i.e. turbidimetry using a Bioscreen and viable counts from broth cultures incubated in waterbaths and a bioreactor. The Bioscreen instrument allows growth, in up to 200 cultures at one temperature, to be monitored simultaneously by

turbidimetry. There were 'edge' effects on the microtiter plates due to desiccation, leading to concentration of media and, presumably, a decrease in water activity. Such data were eliminated, or not able to be used if they formed part of a set of values, e.g., for estimation of generation time by differences in detection time of serial dilutions sets. Due to the operating limits of the Bioscreen (up to 60 °C only), growth rate determinations at higher temperatures (up to 70 °C) required that cultures in shaking waterbaths were used.

More than 300 growth curves were generated, however, only 201 curves were considered of sufficient quality and reliable enough for growth rate estimates based on them to be included in the model fitting. As noted earlier, for pragmatic reasons and to facilitate the modelling the data generated were temperature bounded with T_{min} value constrained to 40 °C and T_{max} constrained to 73 °C (see Fig 2.7a).

The growth rate data were also modelled using Bayesian analysis (*see* Fig 2.7b), which is particularly appropriate for fitting nonlinear models (De La Cruz-Mesía and Marshall, 2006; Li et al., 2012). The prior distributions are based on the growth data of Mtimet et al. (2015) for *G. stearothermophilus* ATCC 12980 within the temperature range from 35°C to 72°C. The aim of this procedure was to improve the estimates of the fitted model for the data derived from the current study using prior information from an earlier study. The process consists of combining prior information for the model parameters

(represented as prior distributions) with observed data from the current study to obtain the posterior estimates of the model parameters.

The data was fitted to Equation 1, in which T is the temperature in Celsius, *r* is the predicted growth rate in hours, and b, c, T_{min} and T_{max} are parameters to be estimated. It was assumed that observed growth rates (r) were Normally distributed as: r~N(r,τ) where *r* is the predicted value from the square root model and τ is a reciprocal variance. To ensure that the *b* and *c* remain positive they were defined as functions of two more variables, *b** and *c** using the functions *b* = exp(*b**) and *c* = exp(*c**). The model was first fitted to the data from the study by Mtimet et al., (2015), but with vague Normal and Uniform distribution priors for the parameters: *b**~N(-1.8, 1); *c**~N(-1.8,1); T_{min}~Unif(0,100); T_{max}~Unif(0, 100). A vague Gamma distribution was used for the observation precision: τ~Gamma (0.001, 0.001). Thereafter, the same model was fitted to the experimental data, but using the posterior estimates from the analysis of the Mtimet et al. (2015) data. Inference was obtained using adaptive Markov chain Monte Carlo (MCMC) sampling methods. The results of the analysis were summarised using Metropolis-Hastings updates.

Figures 2.7a-b show that the growth limits observed in response to temperature are generally consistent with other reports of *G. stearothermophilus* growth, with growth observed at 40 °C and 70 °C, maximum growth rates in the range 58 to 65 °C, and shortest generation times in the range 20 to 25 min (Wind et al., 1994; Ng and Schaffner, 1997; Flint et al., 2001; Burgess et al., 2010; Mtimet et al., 2015). The data of Mtimet et al. (2015) suggest that they observed generation times as short as 15 min

for strain 12890, but that the average generation time at the optimum temperature (~53 – 60 °C) was ~19 min. Figure 2.7b shows the Bayesian-fitted growth rate model using the afore-mentioned priors and leads to an estimate of T_{min} of 41°C and T_{max} of 76 °C, values that are not dissimilar to the estimates and assumptions described above for Eqn. 5.

There is very little unambiguous data for the effect of water activity on *G. stearothermophilus* growth rate, with the data of Ng and Schaffner (1997) being the only systematic study that considered the effect of water activity, albeit indirectly by addition of up to 1% NaCl to “salty carrots”. In that work, the water activity of the “salty carrots’” without added salt was not reported. Nonetheless, assuming that the water activity of unsalted carrots is 0.997, a range of a_{wmin} estimates can be derived from the data of Ng and Schaffner (1997) if it is assumed that the treatment with 0% added NaCl is at or above the a_w optimum for *G. stearothermophilus* growth. From that data (based on the average of 15 data sets for various temperatures and pH for which growth was reported), and after removal of two ‘outliers’ at pH 6.5 at 55 °C and pH 5.5 at 60 °C, the average a_{wmin} calculated was 0.981, but with SD=0.008. Nonetheless, given the variability in the data, and that the Ng and Schaffner (1997) were based on one strain only (ATCC 12890), the data suggests that a_{wmin} estimate calculated in this study (0.983) is credible.

Figure 2.7a-b show that growth rate estimates from turbidimetric methods (OD) were lower than estimates derived from viable counts at the same temperature. In general, faster growth rate were observed in populations

enumerated using viable counts taken from culture grown in waterbaths, or aerobically \using bioreactors.

The wide span of temperature that produces near optimal growth rates (i.e. from 55 to ~70 °C) may present challenges if manipulation of temperature, such as the application of step-change conditions as exemplified by Knight et al. (2004), is to be used as a means of extending run times by slowing the establishment of spore-generating biofilms. The information is nonetheless useful. In their work, Knight et al. (2004), found that inhibition of growth of *Streptococcus thermophilus* in a pilot-scale pasteurisation plant was achieved by implementation of temperature step-changes in heat exchangers, enabling extended run times with fewer cleaning operations.

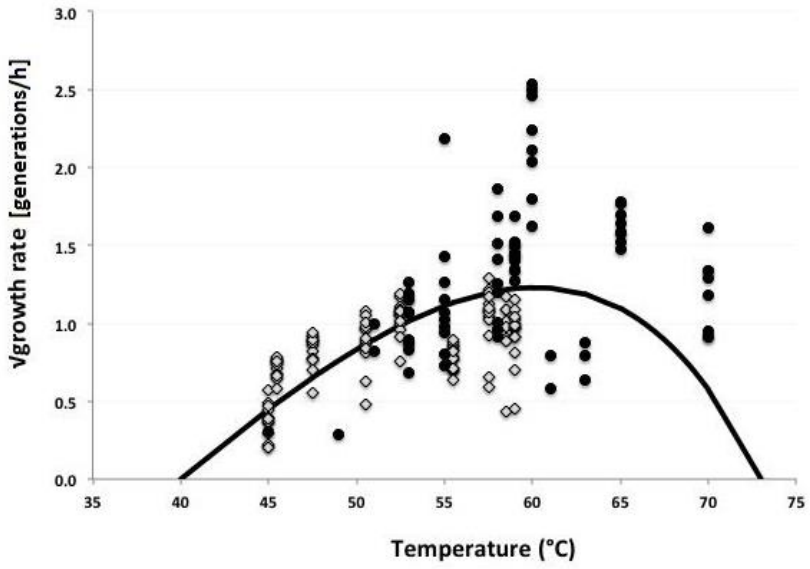
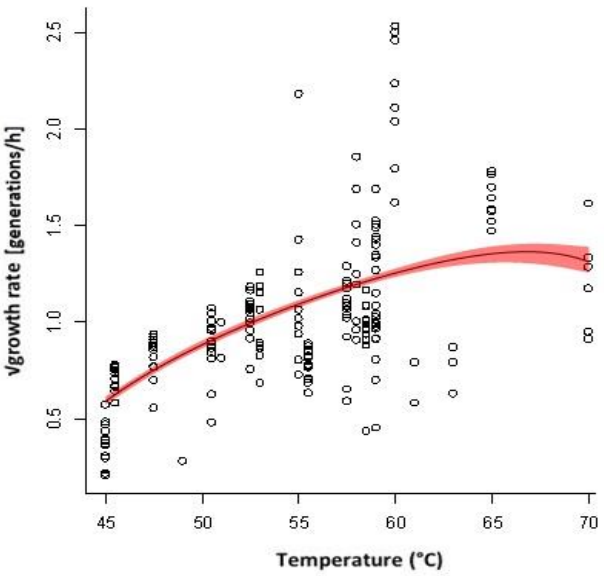


Figure 2. 7a Differentiation of growth rates estimates from viable count data (full black circle) or turbidimetric data (empty diamonds) as a function of temperature.



The summary posterior statistics are shown below.

Parameter	Mean	s.d.	Lower 95% CI	Upper 95% CI
b	0.088	0.007	0.080	0.102
c	0.201	0.066	0.077	0.326
$T_{\min}$	40.986	0.246	40.545	41.455
$T_{\max}$	76.272	1.908	73.141	80.149

Figure 2. 7b. The model (Eqn 1) fitted using Bayesian modelling with Markov Chain Monte Carlo methods. The posterior fitted curve is shown as a solid line. The 95% credible band is shown shaded in red.

As noted previously, growth rate data for all strains were unusually erratic compared to experience in this laboratory with other species and strains of vegetative, non-endospore forming cells. Other workers have described similarly highly variable growth responses (B Searle, pers. comm., 2014, Mtimet et al., 2015). An example from Mtimet et al. (2015) is shown in Figure 2.8.

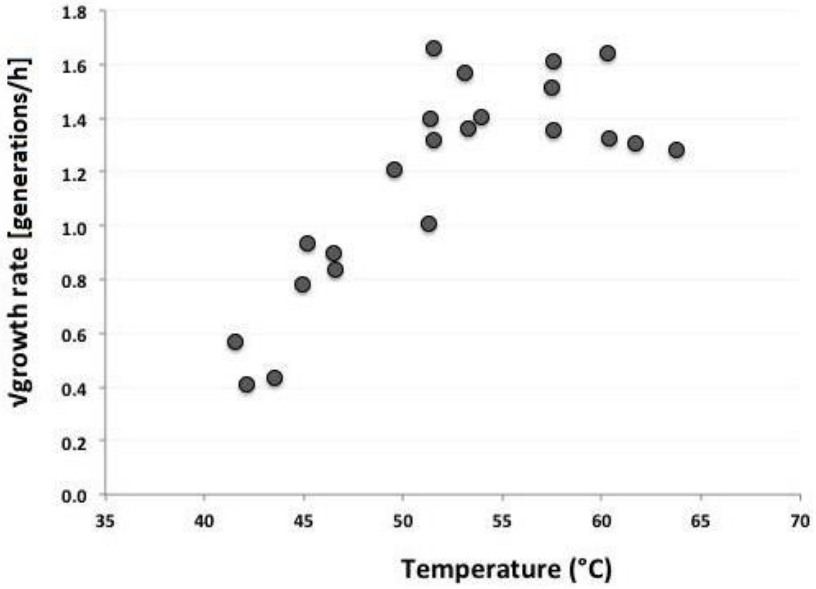


Figure 2. 8 Square root of growth rate data for *G. stearothermophilus* ATCC 12890, from Mtimet et al. (2015) plotted as a function of temperature and showing high variability in observed rates.

Differences between sample means of growth rates can also be influenced by a number of other factors such as genetic variability (Membré et al., 2005), phylogenetic structure in ability to grow at low or high temperatures (Guinebretière et al., 2010), or a particular sensitivity to certain growth media (Carlin et al., 2013). Whether the observed variability is truly a reflection of highly variable growth rates, or due to an inability to accurately measure the growth rates by a variety of methods, requires further study but the contribution of several highly skilled technicians in the generation of the growth rate data, and whose results showed the same variability as the candidate's, suggest that the variability is a property of the microorganisms studied.

In support of this conclusion, examination of the individual growth rate datasets compared to the model fitted to all the data, reveals that the variability in growth rates is not due to systematic differences in the growth of some strains of *Geobacillus* spp. compared to others. Rather, the variability in the growth rates estimates for individual strains is of the same magnitude as the variability in growth estimates among the pooled data at any given temperature.

2.4 Summary and Conclusions

Growth data of *Geobacillus* spp., isolated from Australian milk processing plants, were generated by multiple methods to develop a predictive model for *Geobacillus* growth rate as a function of temperature and water activity. The model was developed to be able to predict the growth of *Geobacillus* spp. under time-varying temperature and water activity conditions. The model was based on growth data from 16 strains.

Within the 201 pooled data there is evidence that the variability in growth rates is not due to systematic differences in the growth of some strains of *Geobacillus* spp. compared to others but is, instead, a characteristic of *Geobacillus* spp. In the work described in this Chapter the growth of *Geobacillus* spp. is within the growth limits expected by reference to the published literature. Although further determination of *Geobacillus* spp. growth rates in dynamic conditions, such as applying different flow rates and temperature cycling is desirable (and is explored in subsequent chapters of

this thesis), the generated mathematical model using various growth media and a wide range of growth temperature and water activity should be useful for predicting and managing the growth of thermophiles in dairy powder plants.

3 Effect of Temperature on Biofilm Formation and Spore Production of *Geobacillus stearothermophilus* within a Model Flow-Through System

3.1 Introduction

As discussed in Chapter 1, *G. stearothermophilus* is the most common thermophilic spore-forming bacterium found in milk powders (Rueckert et al., 2004). As shown in Chapter 2, *G. stearothermophilus* can grow within the temperature range ~40 to ~70 °C, commonly found in pasteurisers and plate heat exchangers in dairy plants (Murphy et al., 1999).

G. stearothermophilus causes problems in dairy powder processing because of its ability to resist high temperature treatments and also because it forms biofilms on milk contact surfaces. As these biofilms develop they begin to produce heat resistant endospores that are released back into the product flow. Development of *G. stearothermophilus* biofilms and spore release into the dairy powder process reduces end-product (milk powder) quality, and the commercial value of the milk powder.

The number of bacteria entering the system with the raw milk is usually low, e.g., ≤50 CFU/mL, (Hill and Smythe, 2012; Kotzekidou, 2014). Both vegetative cells and spores of these bacteria can attach to milk contact surfaces in the process, and eventually form biofilms, particularly at points

such as dead ends, corners of pipes and places that are difficult to clean (Austin and Bergeron, 1995). As discussed in Chapter 1, when spore numbers in end-products increase to more than 10^3 CFU (cells or spores)/g, typically occurring after continuous processing for 16 to 24 h, the plant must be shut down for cleaning to remove the build-up of biofilm microorganisms. Biofilms of thermophiles may also arise from cells/endospores that persisted after the cleaning process. Attachment of thermophilic spore-forming bacteria to pasteuriser plates can occur in the temperature range 45 to 75 °C (Murphy et al., 1999; Scott et al., 2007). *Anoxybacillus flavithermus* and *Geobacillus* species spores have been detected (>1 log CFU/g) in product after approximately 9 h and have been reported to reach levels of up to 10^4 CFU/g in the evaporator after 18 h (Scott et al., 2007). On milk contact surfaces biofilms have been observed to develop concentrations up to 10^6 CFU/cm^2 within approximately 18 h of 'run time', including the presence and production of endospores. Moreover, Murphy et al. (1999) reported that growth of thermophiles was detected in a milk evaporator after a run time of 4 h and increased 100-fold during a 14 h run.

Thermophilic spores are difficult to inactivate and can persist under conditions at which they are unable to germinate. Spores produced in processing lines are able to survive the subsequent heating stages of the milk powder process such as the evaporator preheating steps (75 to 125 °C for 180 s) and spray drying (approximately 70 °C for 30 s) (Bremer, et al., 2009).

To address this problem there has been extensive research (Gibson et al.,

1999; Parkar et al., 2004; Bremer et al., 2006) into appropriate CIP procedures (sanitising), dairy equipment redesign, and production time management, but residual spores in the system, or the few entering the system in the raw milk, are enough to restart the biofilm formation process, limiting the ‘run times’ of milk powder plants throughout the world to only 18 to 24 h.

The success of attachment and time to development of biofilm on stainless steel surfaces may be affected by the length of time the bacteria are in contact with the surface, the temperature of the suspending medium and milk flow rates. The availability of lactose and non-casein protein in solution can also play a role in bacterial attachment (Speers and Gilmour, 1985).

The aim of the experiments described in this Chapter was to observe and quantify the time-course of attachment and proliferation of *G. stearothermophilus* cells and spores using a bench-scale flow-through laboratory “reactor” and to assess the effect of different temperatures and flow rates on cell and spore formation. A bench-scale stainless steel flow through 'reactor', similar to others previously described (Flint et al., 2001; Burgess et al., 2009), was designed and constructed to enable the analysis.

Sterile milk was seeded with *G. stearothermophilus* spore suspensions, and passed through the reactor at constant flow rates. Experiments were performed with various milk flow rates and at temperatures from 45 to 75 °C, relevant to pasteurisers and evaporators in milk powder plants, by immersing

the reactor in a temperature-controlled water bath. Vegetative cells and spores were enumerated in the milk effluent over time to assess the time course of attachment, biofilm formation and spore production and release.

3.2 Materials and methods

3.2.1 Equipment configuration

A custom designed and built bench-top flow through chamber (see schematic in Figure 3.1) was used for all experiments. The apparatus was constructed from grade 316b stainless steel that is widely used for processing equipment in the dairy industry; except for the internal 'coupons' (Grade 304b stainless steel) and tubing (silicone rubber).

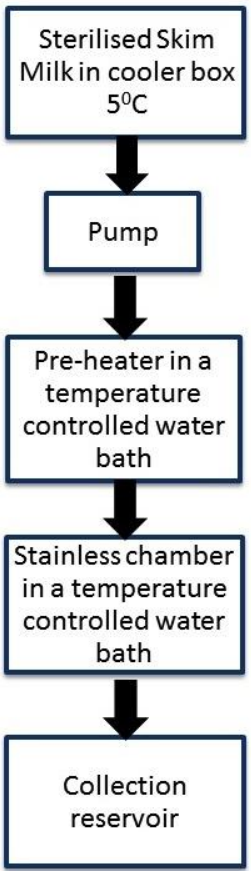


Figure 3. 1 A schematic diagram depicting the flow through system.

After initial trials, the chamber was redesigned so that milk entered the chamber from the top and the effluent exited from the bottom of the other end of the chamber. This was done to achieve better milk flow through the entire chamber and particularly to eliminate dead spots where planktonic microbial growth could occur. A peristaltic pump (Masterflex® L/S® Precision Pump System EW-77800-50 with adjustable speed) was placed between the milk reservoir and the pre-heater section and used to pump the milk through the system at selected flow rates.

The chamber and preheater section were immersed in a water bath (Ratek Instruments Pty Ltd., Australia) for temperature control. The milk reservoir, held in an insulated container, was connected to the preheater section of the system using silicone tubing, which was also used to connect the end of the pre-heater section to the inlet port of the chamber.

The flow through chamber is shown in Figure 3.2, and its components are described here:

i) Main chamber: approximately 21 cm long, with 2.3 cm internal diameter, giving an internal volume of 87 mL and internal surface area of 152 cm^2. The inlet and outlet ports, in the screw caps (see Section iii below), were manufactured from stainless steel.

ii) A stainless steel preheater section of ~ 65 cm length with 4 mm internal diameter was included so that milk entering the chamber would be equilibrated to the set temperature of the water bath. The volume of the

pre-heater section is 8 mL.

iii) Removable screw-on end-caps, with inlet and outlet ports, sealed the main chamber. As noted above, the outlet port was attached to the bottom of the end-cap to prevent any “dead-end” spots in milk flow. Each cap has a 2.5 cm internal diameter with 2.2 cm width. This size allows a space between the removable coupons and the cap, so the flow of the milk through the reactor is not hindered, and to allow milk to flow both above and below the coupons.

iv) Removable coupons: On the inside of the chamber a horizontal ledge was included to allow grade 304b stainless steel coupons to be rested in the main chamber and immersed in the milk flow. A single coupon (21 cm long and 2.2 cm wide) was used in most studies and adds a further 92.4 cm^2 surface area to the inside of the chamber. In other studies a series of coupons (2.2 cm x 2.2 cm) were used to enable the removal of coupons at different times during the experimental run.

v) silicone rubber connecting tubing was used to connect the milk reservoir to the pre-heater; the pre-heater to the main chamber; and the main chamber to the effluent receptacle. The total volume of the tubing was 4 mL.

Prior to each experiment the flow-through chamber, and all tubing, was autoclaved (121 °C; 15 min at 100 kPa).

The experimental set-up is shown in Figure 3.2.

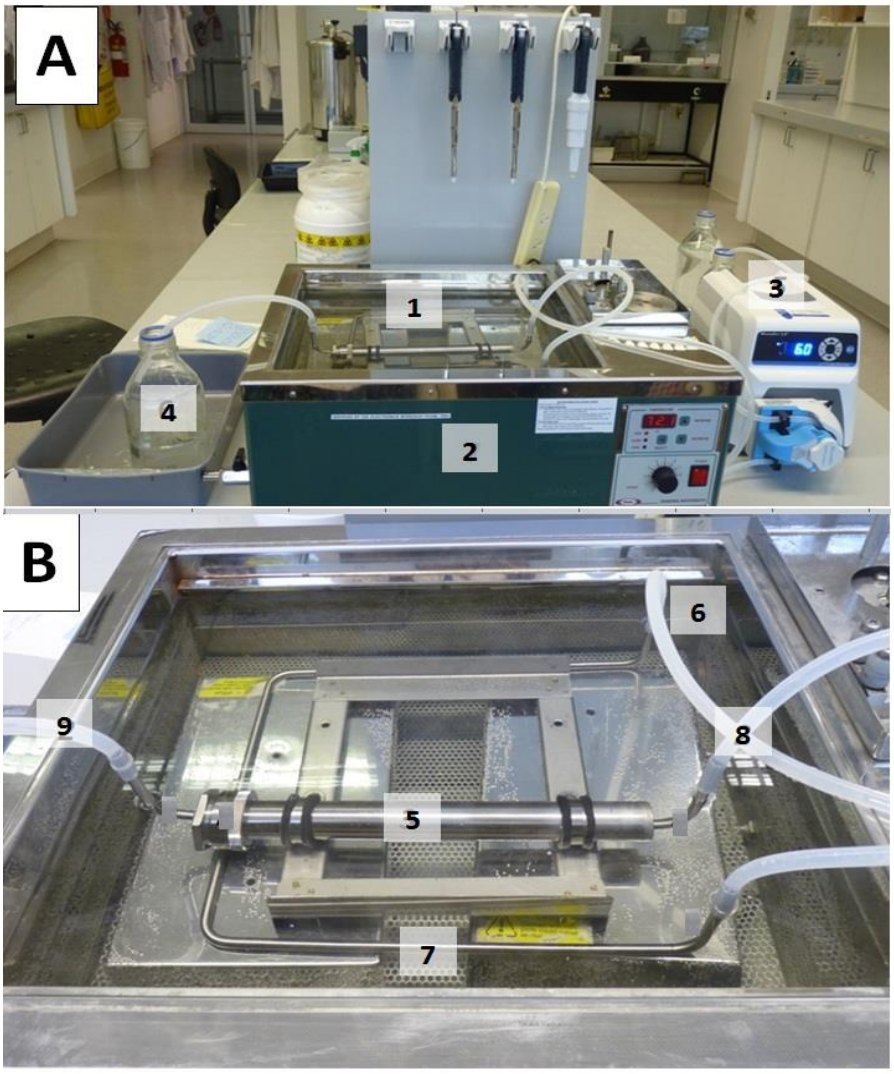


Figure 3. 2 Photographs showing the original set-up of the flow-through chamber apparatus (minor modifications were made subsequently). Image A shows the overall set-up of the flow-through chamber (1) *in situ.* The flow-through chamber sits in a water bath (2) and milk flow is generated by a peristaltic pump (3) and all effluent is collected (4). Image (B) shows the shows the flow-through chamber and preheater section in more detail. The larger stainless steel tube with end-caps (5) is the flow-through chamber. Silicon rubber tubing (6) connects: i) the peristaltic pump (3; Image A) to the stainless steel pre-heater section (7); ii) the preheater to the main flow-through chamber (8); and iii) the chamber to the effluent receptacle (9; and 4 in Image A).

3.2.2 Bacterial strain and growth conditions

G. stearothermophilus strain W14, originally isolated from an Australian milk processing plant, was provided by Dr. C. Pillidge, Dairy Innovation Australia Ltd,, and its identity confirmed by 16s rRNA analysis as described in Chapter 2.

G. stearothermophilus strain W14 was selected for detailed study because its growth rate characteristics are typical of the 16 thermophilic spore forming dairy isolates that were studied (see Chapter 2) and because it produced more reproducible growth characteristics than many of the other 15 isolates. Stock cultures were maintained and resuscitated as described in Section 2.2.1.

3.2.3 Preparation of spore suspensions, and milk

'Sterile' RSM was prepared as described in Section 2.2.4. Spore suspensions used to inoculate the RSM were prepared as described in Section 2.2.3.

After autoclaving at 100° C for 5 min (a milder than usual temperature regime to prevent coagulation of milk proteins), and cooling (1 h at 25 °C), the "sterile" milk was refrigerated at 3 – 5 °C in a standard laboratory refrigerator until needed (up to 12 h). An aliquot (100 µl) of each batch of milk was plated on to TSA plates, and incubated at 55 °C for 18 h to check for microbial contamination after autoclaving the milk and before inoculation with the *G. stearothermophilus* W14 spore suspension.

For each experimental “run”, at least eleven 2L batches of RSM, and one 5L batch were prepared. Each experiment required 20 - 30 L of RSM.

3.2.4 Methodological variations in system application

In all trials, cold (4 °C) RSM containing controlled levels of *G. stearothermophilus* spores was pumped through the flow-through chamber *via* silicone tubing and the stainless steel pre-heater section at controlled flow rates. In studies of the time-course of attachment of cells/spores to the stainless steel surfaces, and subsequent proliferation inside the flow through reactor, two methods were used:

i) Continuous inoculation

A spore suspension (4 mL containing ~10^5 CFU/mL; Section 2.2.3) was added to every batch of milk used in the experiment. This inoculated milk was kept refrigerated and enumerated prior to use on the same day of the experiment.

ii) Pulsed inoculation

A spore suspension (2 mL containing ~10^5 CFU/mL; Section 2.2.3) was added to 1 L of milk and fed through the flow-through system for ~50 minutes. For the remainder of the duration (~24 h) of each ‘pulsed inoculum’ experiments, only ‘sterilised’ milk, un-inoculated RSM (20-30 litres per experiment) was pumped through the system.

In either approach, samples of the milk leaving the chamber (the ‘effluent’)

were taken every 5 min for the first hour and every hour thereafter to enumerate spores and vegetative cells. Prior to sampling the effluent, the sampling port was sprayed with 70% alcohol and let run to waste for several seconds before samples were taken. The system was run for 15 to 20 min before taking the first sample (T_0) to ensure that the desired temperature had been obtained and to minimise the possibility of air bubbles inside the chamber and tubing. Additionally, the pH of the effluent milk was determined as a function of time in all experiments. Temperature loggers (iButton, Maxim Integrated, San Jose, California, USA) were placed both in the water bath and the polystyrene cooler-box containing the milk reservoir to continually monitor temperatures during experiments.

3.2.5 Experimental variables to investigate kinetics of attachment, biofilm formation and spore production

A series of experiments were undertaken using the flow through chamber apparatus. Variables included i) the temperature in the main chamber/water bath, ii) the flow rate of the milk through the system, iii) levels of spores and vegetative cells in the influent milk used in experiments, and iv) whether the influent milk was continuously dosed with *G. stearothermophilus* W14, or whether only an initial ‘pulse’ of spores was applied.

Experiments were conducted for 16 to 24 h, consistent with maximum run times achieved in commercial milk powder processing plants. Flow rates applied for most experiments were 20 mL/min. In some experiments the effects of higher, or lower, flow rates were explored.

Tables 3.1 and 3.2 (*see* Section 3.3, 'Results') show the specific experimental conditions for each of the 11 trials conducted.

3.2.6 Microbial enumeration of milk fouling on internal surfaces

In addition to monitoring cells loads in the effluent, at the completion of experiments, the flow-through chamber was dissembled and vegetative cells and spore counts per cm^2 were determined on various internal surfaces of the reactor and also the preheater section by swabbing. Swabs were taken using sterile cotton swab sticks at several sites including the coupon upper surface and lower surface (area swabbed 46.2 cm^2), pre-heater inlet and outlet (area swabbed 7.536 cm^2), internal surfaces of the chamber (total surface of 152 cm^2) and inside cap (area swabbed 11.38 cm^2).

After swabbing the nominated surface, the end of the swab was cut off and immersed in 9 mL sterile distilled water in a 10 mL sterile disposable centrifuge tube containing 2.8 mm ceramic beads. The tube was vortexed for 1 min, and 1 mL of the supernatant transferred into each of two 1.5 mL Eppendorf® tubes. The first tube was used for spore enumeration, i.e., after exposure to boiling water (100 °C) for 30 min. The second tube was used to enumerate total vegetative cells and spores. Serial dilutions (1:10) of both suspensions were prepared in sterile 0.1% bacteriological peptone water and 100 µl aliquots of appropriate dilutions were plated on TSA plates and spread evenly with disposable L-spreaders (Copan, Brescia, Italy). The plates were incubated at 55 °C for 24 h. The total viable count and spore count were

determined as described in Section 2.2.

3.2.7 Cleaning procedure

The components of the system were sterilised and cleaned after each experimental run by autoclaving (121 °C, 15 min), followed by soaking in strong detergent ('Pyroneg', 10 g per L tap water). Manual cleaning was done as appropriate and feasible (e.g., the narrow diameter and bends of the pre-heater section made mechanical cleaning difficult) and using the cleaning procedure recommended by Bylund (1995) involving: i) warm water rinsing, ii) circulation of 1.5% (w/v) sodium hydroxide for about 30 min at 75 °C, iii) circulation of 0.5% (v/v) nitric acid solution for about 20 min at 70 °C, iv) post treatment rinsing with cold water.

3.2.8 Analysis of growth kinetics

To analyse the growth kinetics of *G. stearothermophilus* during the flow through experiments, linear regression analysis of the growth curve data was used to estimate exponential growth rates. A straight line was fitted to points that best represented the exponential growth phase.

Doubling time ('*DT*'; h) was calculated by dividing the slope of the regression line '*m*', by 0.301 (equivalent to $\log_{10}2$), i.e.

$$DT=0.301/m \qquad \textbf{Equation 6}$$

Where *m* = slope of regression line fitted to data in the exponential growth phase.

Lag time was defined as the time at which the regression line through the exponential part of the growth curve equals the initial spore concentration:

$$Lag\ time = (logN_{(initial)}-c)/m \qquad \textbf{Equation 7}$$

Where $logN_{(initial)}$ = log CFU/mL at time = 0, *c* = intercept of the regression line through the exponential growth phase and *m* is the slope of the regression line through the exponential growth phase.

Growth rate (Gen/h) was calculated as the reciprocal of generation time:

$$Growth\ rate = 1/(Doubling\ Time) \qquad \textbf{Equation 8}$$

3.3 Results

The flow through chamber (Figures 3.1 and 3.2) was designed and used to understand better the dynamics of *G. stearothermophilus* growth, attachment, and spore formation under conditions similar to those in a dairy powder plant. To simulate the process of attachment of thermophilic spore-formers from milk to the stainless steel surfaces and their eventual release into milk flowing through the system, milk inoculated with spores of *G. stearothermophilus* W14 was pumped through the chamber. Vegetative cells and spores in the

milk effluent from the chamber were enumerated periodically to be able to estimate times for attachment, biofilm formation, and production and release of new cells and new endospores into the milk flow from the biofilm formed. Details of the experiments undertaken and the results obtained are summarised in Tables 3.1 and 3.2.

Table 3. 1 Summary of experimental conditions and the time course of both *G. stearothermophilus* vegetative cell and spores production during the various trials

Experiment Number	Temperature (°C)	Flow Rate (mL/min)	Time to vegetative cell growth (h)	Time to spore load increase (h)	Approximate difference between vegetative cells and spores (log CFU/mL)	Doubling time vegetative cells (min)	Doubling time spores (min)	√(Growth Rate [1/h])
Before Equipment Modification								
1	50	10	2.5	11.9	2.56	61	131	0.99
2	55	5	1.5	11.3	2.58	71	63	0.92
3	55	20	3.6	n/a	n/a	34	n/a	1.34
Continuous Inoculum System								
4	45	20	16.1	n/a	n/a	27	n/a	1.48
5	50	20	7.5	>20	2.8	77	n/a	0.88
6	55	20	5.0	1.9	2.97	38	32	1.25
7	65	20	2.9	5.3	1.90	72	77	0.92
8	65	20	1.7	5.5	2.86	43	37	1.19
9	65	40	2.6	7.3	3.30	24	25	1.59
10	70	20	>24	n/a	n/a	n/a	n/a	n/a
11	75	20	n/a	≥24	n/a	n/a	n/a	n/a
Pulsed Inoculum System								
12	55	20	≥22	n/a	n/a	n/a	n/a	n/a
13	55	10	5.2	>24	n/a	60	n/a	n/a
14	60	20	14.4	>21	n/a	29	n/a	1.44
15	65	20	1.6	6.8	2.15	42	n/a	1.19
16	67.5	20	8.7	4.1	3.75	35	40	1.30

Table 3. 2 Average of total cells in the flow-through chamber and cells and spores of *G. stearothermophilus* W14 produced per hour near the

end of each of the various flow-through trials.

Experiment Number	Temperature (°C)	Flow Rate (mL/min)	Milk 'Residence Time' in Chamber (min)	Average total cells in chamber	Average log cells produced *	True generation time (h) at experiment end	Run Tim (h)
Before Equipment Modification							
1	50	10	10	$2.81x10^8$	7.95	3.1	16
2	55	5	20	$9.93x10^8$	9.06	2.9	20
3	55	20	5	$4.37x10^7$	7.36	2.9	12
Continuous System							
4	45	20	5	$1.75x10^4$	3.39	2.4	24
5	50	20	5	$7.63x10^5$	3.74	2.3	24
6	55	20	5	$8.08x10^5$	5.21	2.5	18
7	65	20	5	$5.46x10^6$	5.08	2.7	18
8	65	20	5	$5.65x10^5$	4.47	3.1	24
9	65	40	2.5	$1.5x10^6$	5.17	3.5	30
10	70	20	5	nd	nd	nd	24
11	75	20	5	nd	nd	nd	24
Pulsed System							
12	55	20	5	$9.83x10^1$	0.8	3.5	24
13	55	10	10	$2.42x10^6$	4.35	3.4	24
14	60	20	5	$6.13x10^5$	3.85	2.8	24
15	65	20	5	$3.48x10^7$	6.13	3.5	24
16	67.5	20	5	$9.0x10^7$	6	3.4	24

[×]Growth rate was determined by considering the total number of viable cells produced in the last hour of the "run" (concentration in the chamber per hour) and the number of cells attached to surfaces in the chamber. The calculations are described in Section 3.3.4.

3.3.1 Equipment redesign

In initial experiments (experiments 1 – 3) it was noted that milk had coagulated in the chamber and end caps when the system was disassembled for swabbing (see Figure 3.3). Accordingly, the results and analysis of results from those experiments, while presented in Tables 3.1 and 3.2, are considered unreliable due to the confounding effect of poor flow and growth in the milk itself, rather than from biofilms that had formed. Those results are not considered in further detail and are included for completeness only.

As a result of those observations, however, a number of modifications were made to the original design of the flow through chamber. The preheater section was added after initial studies showed it was needed to ensure the milk entering the chamber was at the intended temperature. A further modification of the chamber, after conducting the three initial experiments, was done to prevent ‘dead spots’ in the chamber and to prevent milk coagulating in the lower section of the chamber as was observed in initial trials (Figure 3.3). The chamber was redesigned so that the milk entered the top of the chamber and was drawn off from the bottom of the chamber, to achieve more consistent milk flow through to the entire chamber. Spaces at either end of the coupons (i.e. between the coupons and the end caps) were included to allow better milk flow through the entire chamber. Subsequent to this modification, clumps of coagulated milk were not observed in the chamber upon completion of the trial.

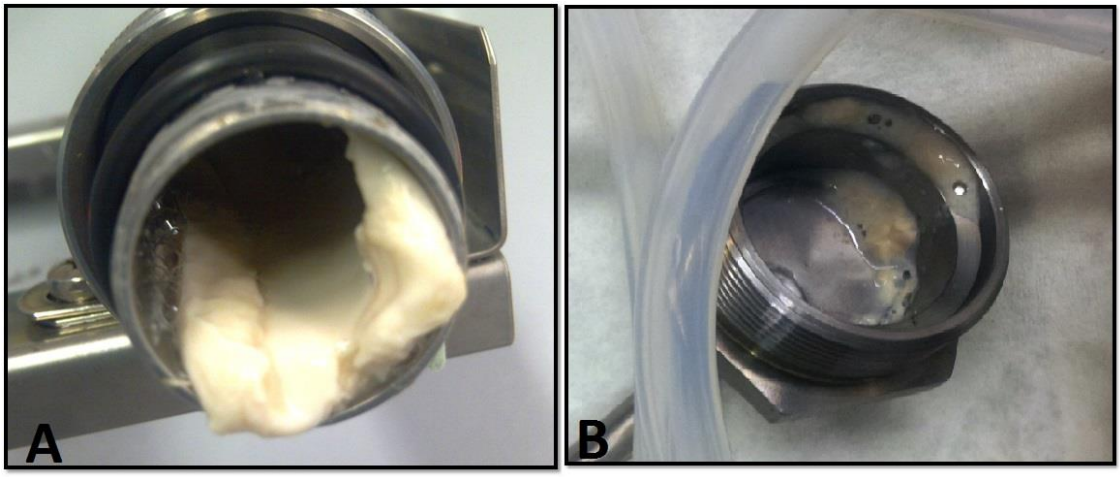


Figure 3. 3 Milk fouling/coagulation observed in the chamber after initial trials and before modification of the flow-through chamber design. A) chamber and B) lid.

3.3.2 Levels of spore inocula used in trials

In the flow-through experiments, milk was inoculated with spores of *G. stearothermophilus* W14 (see Section 3.2.3). To simulate commercial conditions, spore levels were set as low as possible but to also enable spore attachment leading to biofilm development. Initial trials were undertaken with spores added to all milk pumped through the system, but it was recognised that a limited addition of spores would better facilitate interpretation of the results, i.e., without the confounding influence of continuous external addition of spores to the milk. Ultimately, an initial inoculum level of ~100 CFU/mL was found to be adequate for most experiments, including the 'pulsed' inoculum experiments. Thus, whereas in the 'continuous inoculation' experiments several million endospores were added to the system, in the pulsed

experiments, ~100,000 endospores (1,000 mL x 100 endospores/mL) in total were added to the system.

The overall strategy of the experiments was to enumerate spores and vegetative cells of thermophilic bacteria (assumed to be *G. stearothermophilus* W14) in effluent milk with the aim of estimating the time required for the spore levels to increase to levels higher than those in the influent milk, as a function of temperature and flow rate, and as an indication of the kinetics of endospore attachment and biofilm formation and release.

3.3.3 Development and release of cell and endospore during 'continuous' and 'pulsed' inoculum trials

Growth kinetics in the flow-through system were studied at temperatures in the range 45–75 °C, i.e., temperatures commonly found in pasteurisers and plate heat exchangers (Murphy et al., 1999) in dairy processing plants. Most experiments were conducted for 18 to 24 h, consistent with maximum run-times possible in commercial powder processing plants.

Bacterial growth was detected as increased vegetative cells and spore counts in the effluent within the temperature range 45-67.5 °C. The kinetics of increase of vegetative cell and spores introduced into the system are summarised in Table 3.1. The optimum growth temperature in the flow-through experiments seemed to be near to 65 °C (compared to the ~60 °C optimum observed with static, or shaken, cultures – see Chapter 2), although there is considerable variability in the data. Temperature affected the

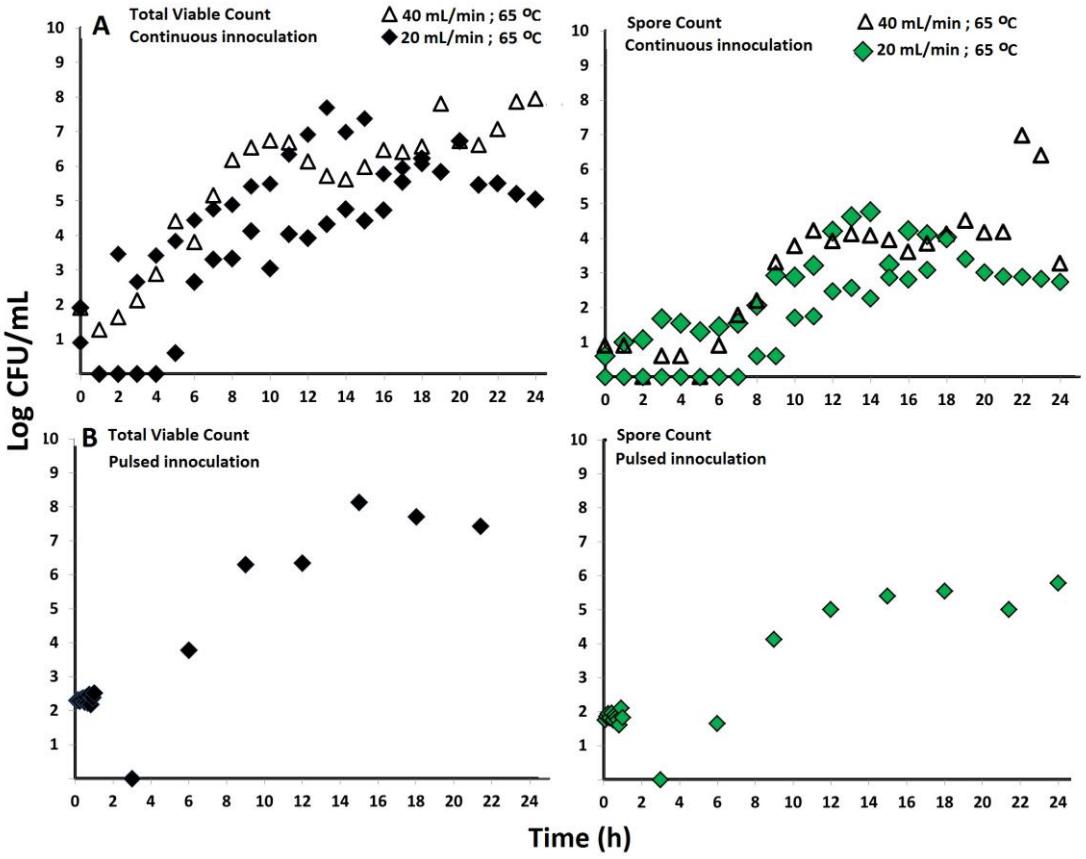
vegetative cell growth which becomes apparent after a 3 h run time (pulsed-system) or a 2 h run time (continuous-system).

In the 18 to 24 h continuous inoculum experiments, the time to emergence of new vegetative cells at 65 °C was ~2 to 3 h but required 5-7 h for spore levels to increase above the level in the inoculum. In experiment 9 (Table 3.1) at 65 °C emergence of spores above the background level took longer time (7 h) than in other experiments at 65 °C (5 h).

Typical growth curves for both continuous inoculation and pulsed inoculation are shown in Figure 3.4 and illustrate the differences in time to appearance of spores and vegetative cells in the milk effluent and also illustrate the ratio of spores to vegetative cells, even though spore suspensions were initially inoculated. The ratio during the exponential growth phase is typically around 100:1 to 1000:1 vegetative cells to spores, as exemplified in Figure 3.4 and summarised in Table 3.1.

At 65 °C, in the pulsed system vegetative cells started to increase above the inoculum level after ~1.6 h and spores started to increase above the maximum level after ~ 6.8 h. Experiment 14 (Table 3.1), at 60 °C, showed a much longer time for both vegetative (14.4 h) and spores (>21 h) to increase above inoculum levels, for reasons that are not currently apparent. The time to increase cell and spore density above the inoculum level was generally temperature dependent, and took longer at lower temperatures, although there were anomalous results (e.g. experiment 12 and 15, pulsed, 55 and 65

°C) in which estimated generation times were less than those observed at temperatures closer to 60 °C.



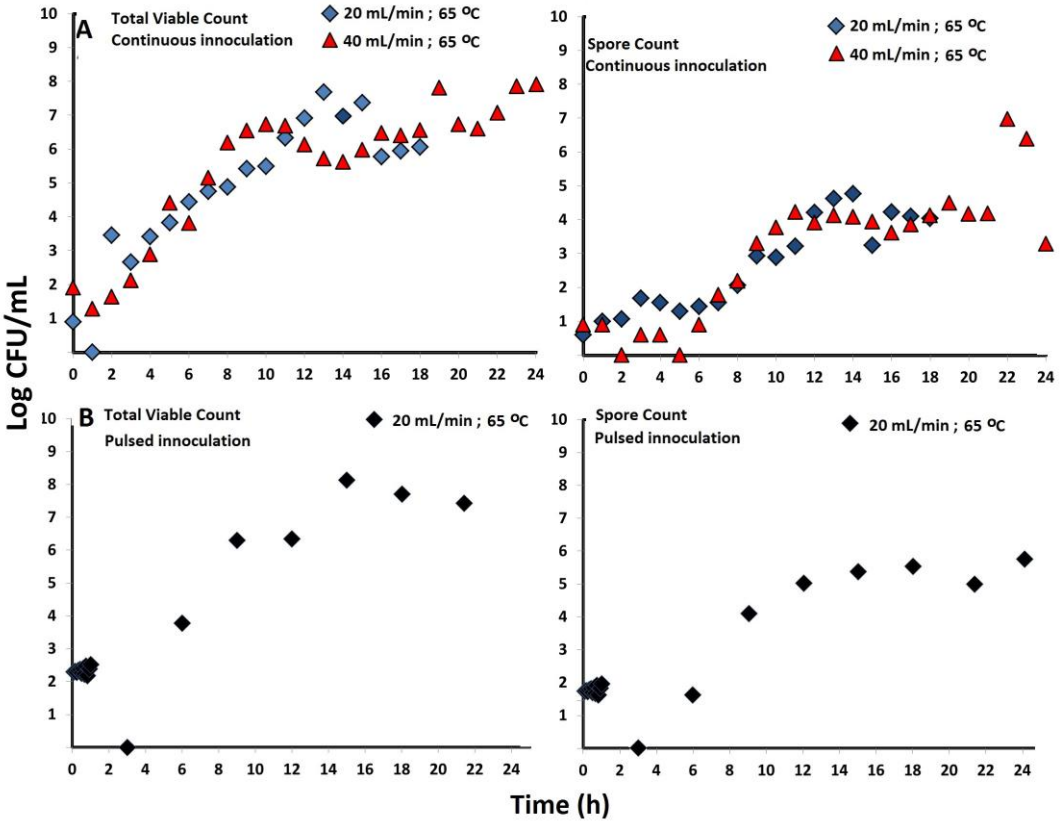


Figure 3. 4 Comparison of evolution of spores and vegetative cell numbers in milk effluent from a flow through chamber at 65 °C either A) dosed continuously with ~100 spores/mL in the influent milk, or B) dosed with an initial pulse (for 60 min) of ~100 spores/mL in the influent milk. The upper figure also compares the kinetics of vegetative cell and spore increase as a function of milk flow rate and reveals that flow rate has very little influence on kinetics in the redesigned flow through chamber.

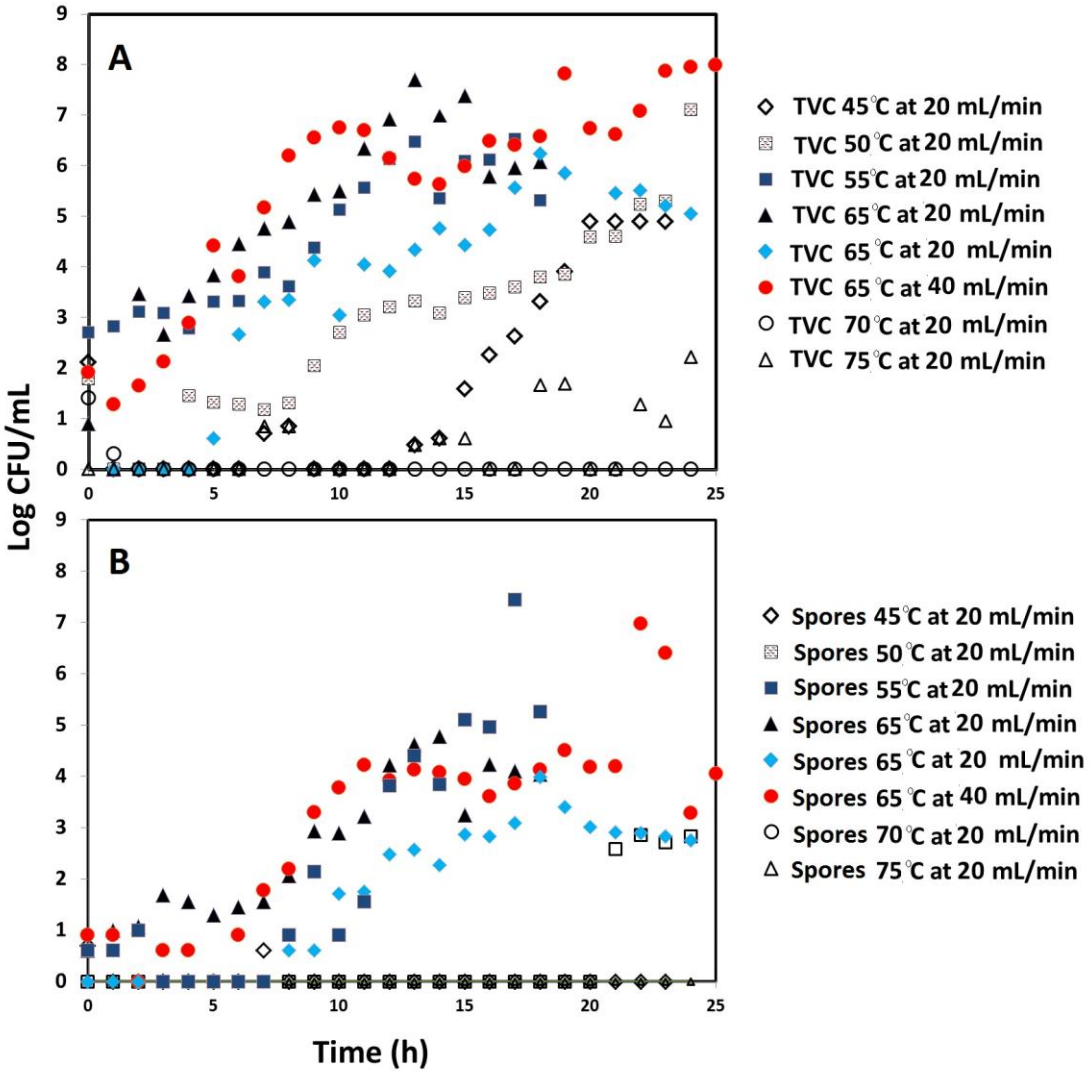


Figure 3. 5 The effect of temperature and time (24 h) on the number of A) vegetative cells and B) spore counts of *G. stearothermophilus* in the effluent from the reactor system when continuous spore applied.

Figure 3.5 illustrates the effects of temperature and time (24 h) on the number of vegetative cells and spores of *G. stearothermophilus* W14 in the effluent from the reactor system when a continuous spore inoculum was applied. The rate of feeding of spores into the system was < 100 CFU/mL. From Figure 3.5

and Figure 3.6 it can be seen that new spore formation began only after 7 h operation at 65 °C but took progressively longer at lower temperatures (60 and 55 °C; Figure 3.6), as might be expected from the slower growth rates (described in Chapter 2). From Figure 3.5, it is also suggested that a temperature of 70 or 75 °C is less optimal than 65 °C for the fastest production of new cells and spores in the flow through chamber.

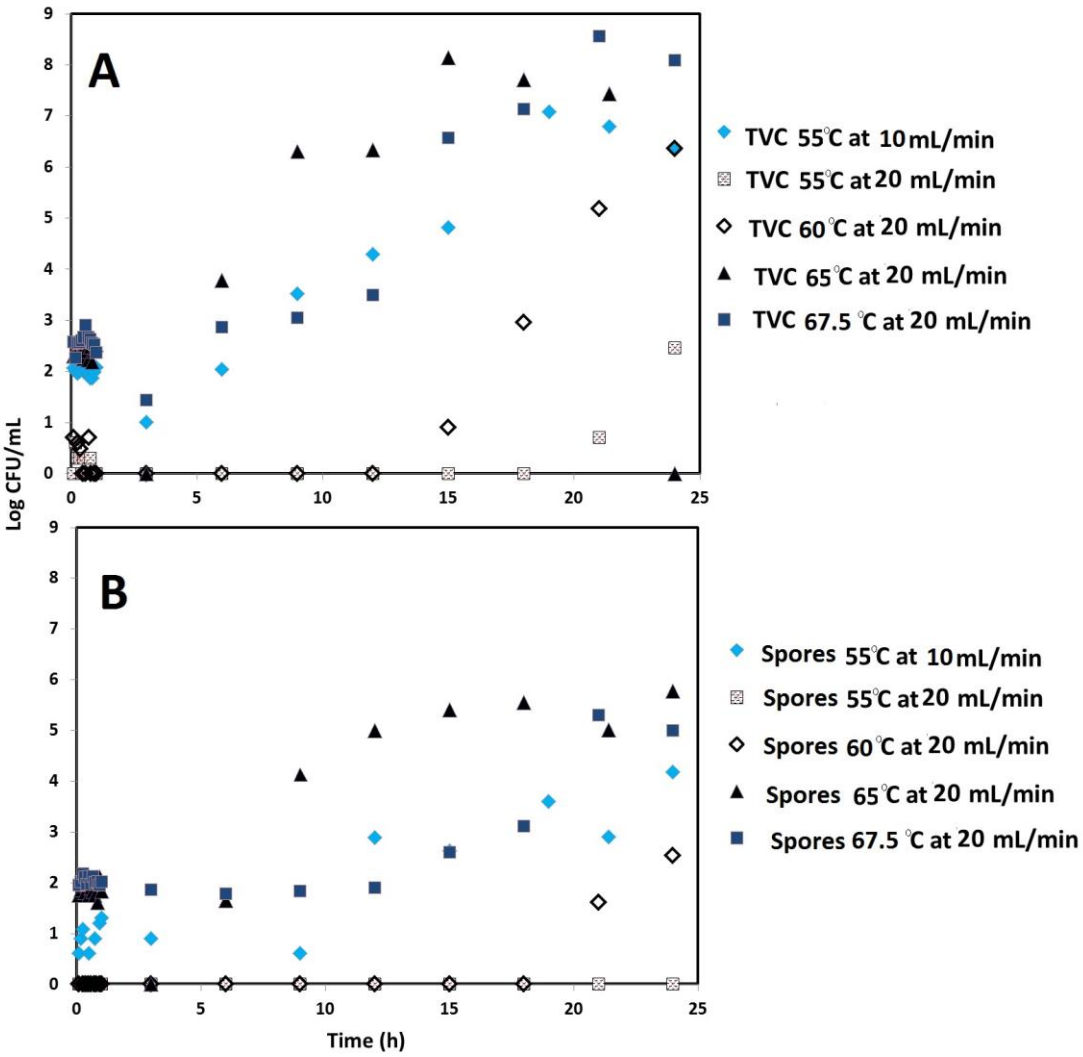


Figure 3. 6 The effect of temperature and time (24 h) on the number of A) vegetative cell and B) and spore counts of *G. stearothermophilus* in the effluent from the reactor system when pulsed spore inocula were applied.

Figures 3.4 - 3.6 show that at near to the optimum temperature for growth rate (60 to 65 °C), *G. stearothermophilus* W14 in the effluent reached cell densities of 10^8 CFU/mL during a 24 h run, whereas no growth was observed within 24 hours at temperatures above 70 °C or below 55 °C. Furthermore, the Figures show that the spore concentration in the effluent achieved maximum levels of 10^5 CFU/mL at temperatures near to the optimum of 60 – 65 °C, and reinforces the observation that spore concentrations in the effluent were typically 100 to 1000- fold less than vegetative cells.

3.3.4 Effect of milk flow rates on growth kinetics

Several flow rates were applied in different 'runs' to assess potential effects of flow rates (i.e., 5, 10, 20, and 40 mL/min) on spore attachment and biofilm development.

Given the volume of the flow-through chamber (~87 mL), pre-heater section (~9 mL) and the connecting tubing (~4 mL), the milk in the system would be expected to be exchanged approximately every 20 minutes if the flow rate of the milk is 5 mL/min, every ten minutes if the flow rate is 10 mL/min, every 4 to 5 min if the flow rate is 20 mL/min and every 2 to 3 minutes if the flow rate is 40 mL/min. All of these flow rates were trialled, although only flow rates of 10 to 40 mL/min were trialled in the redesigned flow-through chamber.

Given the expected growth rates (see results in Chapter 2) of 15-20 minutes per generation at optimal temperature, these flow rates would prevent planktonic growth of added *G. stearothermophilus* W14 being the source of

the observed increases in vegetative cell and spore loads in the effluent, unless there were dead spots in the system, i.e., even at the optimal temperature the dilution rate exceeds the expected growth rate.

Vegetative cells and spores were enumerated in the effluent and results are summarised in Table 3.2. The increase in number of cells or spores over time from the effluent, and at different flow rates, indicated that the rate of vegetative cell growth and spore production were not noticeably affected by flow rates applied in the redesigned flow chamber, neither in continuous or pulsed inoculum trials (See results in Table 3.1 and 3.2).

Following from the above, given the hypothesis that the spores and cells in the effluent arise from cells and spores released from a biofilm, the true rate of growth/production can only be calculated if the total number of cells attached to the internal surfaces of the chamber is known at some fixed point in time, and can be related to the concentration of cells in the effluent stream at the same time. This is because the apparent growth rate is slower due to the effects of dilution from the flowing milk.

Thus, measurement of cell densities inside the chamber would need to be made periodically to estimate ‘true’ growth rates, i.e., allowing for the dilution effect. The available data, however, enable estimates of cell concentration within the chamber only at the end of the experiment. This procedure was exemplified by Flint et al. (2001). From estimates of the total number of cells released into the milk effluent in the last hour of experiment, and the number

of cells attached to the internal surfaces of the chamber at the end of the experiment, the rate of production of new cells from the attached cells can be made. This was done for a number of experiments for which the necessary data were available. While the estimates ranged widely, a mean estimate of 9.8 times as many cells produced in the last hour (calculated from the flow rate and cell concentrations in the milk effluent) compared to the total number of cells attached to the inner surfaces of the chamber, was obtained. This rate of cell/spore production corresponds to ~3.3 generations of growth from biofilm in one hour. In turn, this leads to an estimate of a generation time of ~18 min, in the optimal temperature range (60-65 °C), and is very similar to the shortest generation time for *G. stearothermophilus* estimated by Flint et al. (2001) using a similar flow-through.

3.3.5 Internal surface contamination

To further assess the hypothesis that the cells in the effluent arise from detachment of cells from a biofilm, rather than growth in the milk resident in and flowing through the chamber, the internal surface of the lid, the inside of the reactor chamber, and upper and lower surfaces of the coupon were swabbed to enumerate vegetative cells and spores attached to those surfaces after several runs, involving both pulsed and continuous spore-feeding methods. The results are presented in Figures 3.7 and 3.8.

Fluctuations in the level of attached cells of *G. stearothermophilus* W14 were observed under a range of spore addition conditions in different parts of the flow through equipment. The reasons for these fluctuations are unclear but

may arise from differences in the steel type, effective flow rates and sheer forces, irregularities in the steel surfaces etc., but equally from stochastic process that determine where spores attach, germinate, and begin to replicate.

In the continuous inoculum system (Figure 3.7) at 65 °C (near to the optimum temperature for cell and spore production) both vegetative cell and spore counts were highest on the upper surfaces of the coupon (A), inside surfaces of the chamber (B), and inside the cap (C). The spore count on the upper side of the coupon upper side was 10^7 CFU/cm^2, whereas the underside showed much lower contamination of 10^2 CFU/cm^2. The inside of the chamber and cap had spore densities of 10^5 CFU/cm^2 regardless of the milk flow rate. vegetative cell counts of 10^7 CFU/cm^2 were observed on the inside chamber, inside of the cap and on either side of the coupons.

Using the pulsed inoculum system (Figure 3.8), the highest counts both for vegetative cells and spores were found on both upper and lower surfaces of the coupons (A), the inside of the chamber (B) and the cap (C). Slightly higher counts were found inside the chamber after 60 °C incubation; especially with the spore counts on the coupon upper side and underside, inside of chamber and the cap. The spore counts were 10^5 CFU/cm^2, whereas the vegetative cell counts on the coupon upper side and underside were 10^5 and 10^7 CFU/cm^2 respectively. This again seems to reinforce the level in proportion of vegetative cells to spores in the system and release from the system.

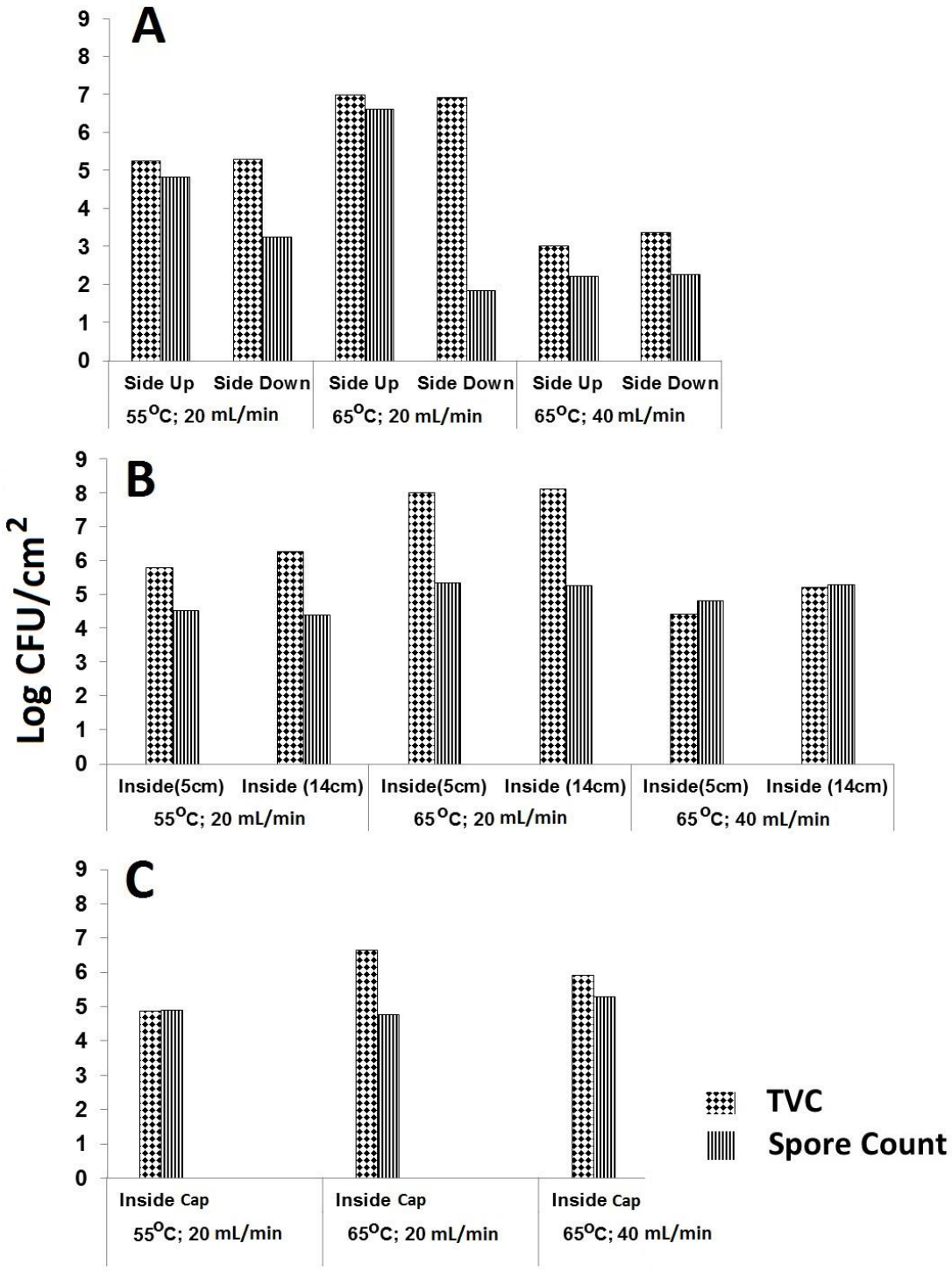


Figure 3. 7 Each plot presents the density of vegetative cell and spore populations of *G. stearothermophilus* after 24 hours of operation of the flow through system and continuous addition of spore inocula: A) Both sides of the coupon within the chamber; B) inside of the chamber; C) the inner cap section of the reactor.

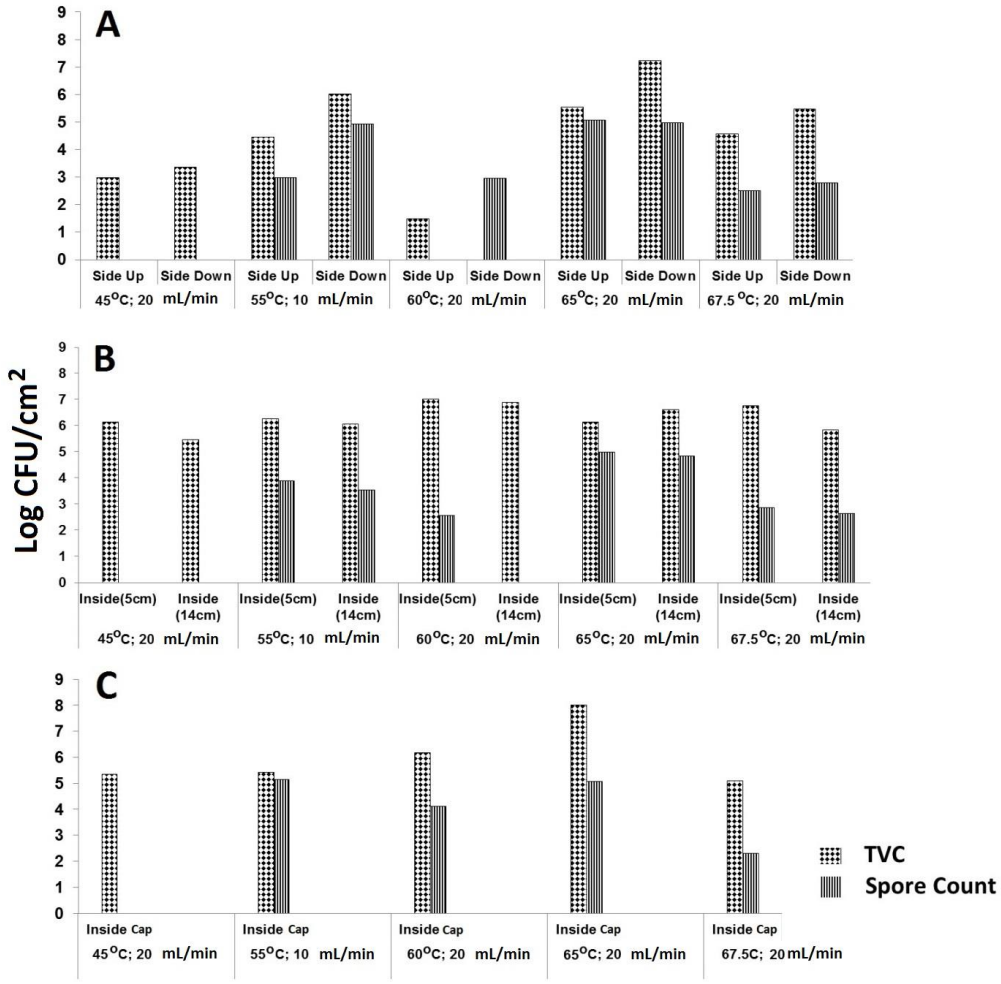


Figure 3. 8 Each plot presents the density of vegetative cell and spore populations of *G. stearothermophilus* after 24 hours of operation of the flow through system within the chamber : A) Both sides of the coupon; B) inside of the chamber; C) the inner cap section of the reactor, under conditions of pulsed spore inoculation for one hour.

3.3.6 Modelling of the effect of temperature on growth rate

The square root of observed growth rates from the flow-through system and other data generated using Bioscreen C, waterbaths, and bioreactor (Appendix 3) is compared in Figure 3.9. The flow through data seems to produce rates, on average, faster than estimated from the static cultures.

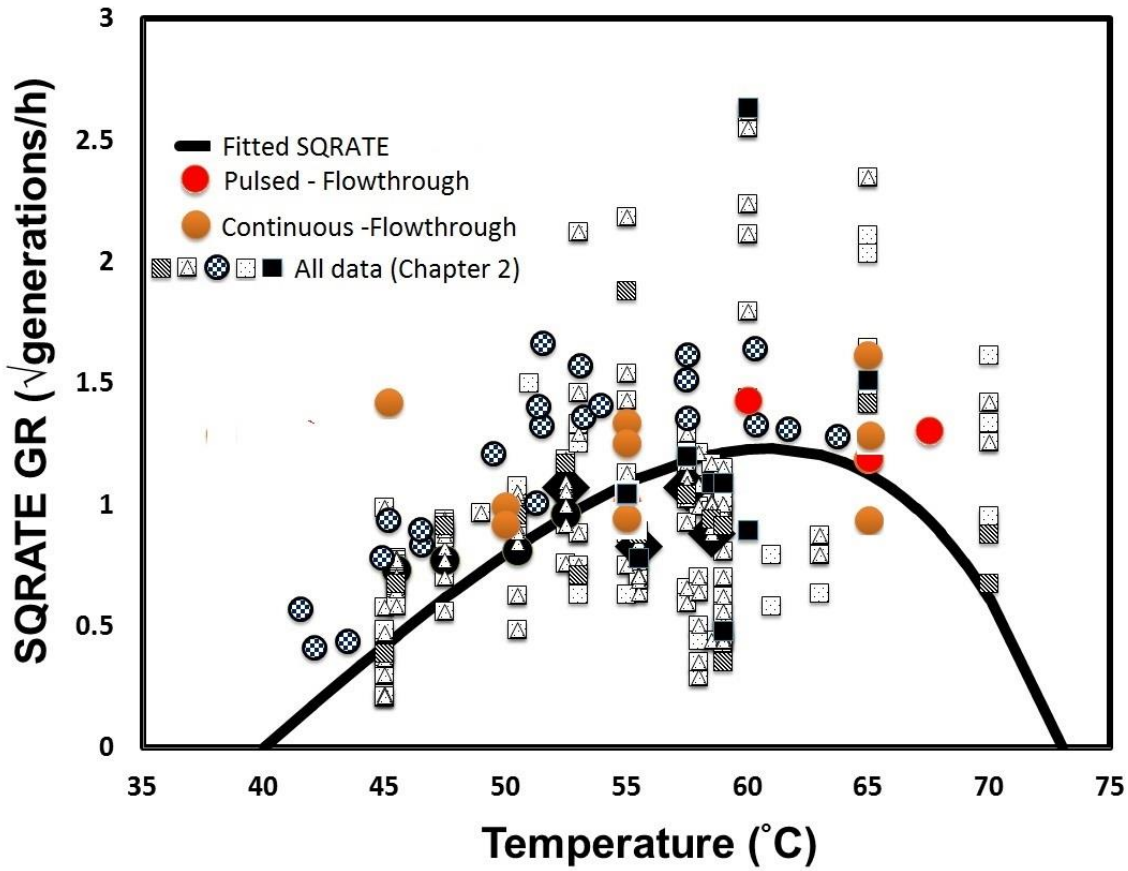


Figure 3. 9 Growth rates of *G. stearothermophilus* W14 generated from flow through experiment, both pulsed and continuous system, compared to a predictive model (Equation 4) for the effect of temperature on growth rate based on studies in liquid media, and also showing growth rate estimates for all date described in Chapter 2 for all strains.

3.4 Discussion

In this study, attachment and growth of *G. stearothermophilus* spores from milk to stainless steel surfaces in a flow-through system was investigated over a range of temperatures from 45-75 °C. The results suggested rapid attachment of *G. stearothermophilus* spores and germination and proliferation at temperatures from 45 to 70 °C.

The temporal changes in vegetative cells and spore numbers from the various experiments strongly suggest the formation and development of biofilms of *G. stearothermophilus* W14 on the internal surfaces of the flow-through chamber during the experimental trials.

Compared to spore levels in the influent milk, the concentration of vegetative cells in the effluent flow started to increase after 2-3 h at 65 °C, whether using a continuous inoculation or pulsed inoculation (Table 3.1). This suggests that attachment, germination and proliferation occur quite rapidly, whereas new spore development was detected only after 6 to 9 h at 65 °C (Table 3.1) despite that spores only were used as the inoculum. Given that only a small fraction of the milk flowing through the chamber would be in contact with the chamber surfaces if the flow were laminar, any spores in the milk would only have brief contact with surfaces; this suggests that the probability of attachment of spores of *G. stearothermophilus* is high, or that the equipment itself provided the inoculum. However, unlike powder processing plants, it was possible to autoclave the flow-through reactor components using conditions that ensure inactivation of spores of *G. stearothermophilus* on surfaces. Thus, growth in the system is expected to have arisen from spores in the effluent

that attached to the internal surfaces, germinated and began to develop biofilms. That this attachment and proliferation occurred even in experiments using an inoculum “pulse”, of low inoculum number in flowing milk, further reinforces the suggestion that attachment is rapid and likely if contact occurs.

As expected, at 45 °C, the time required for production of new vegetative cells and spores was slower requiring 12 and 24 h (Table 3.1; except for #10), respectively, before cells and spores were detectable in the effluent milk. Different milk flow rates produced no significant differences in the time for the generation of vegetative cells or spore. Swabbing different parts of the equipment at the end of runs at 45 °C, showed the early stage biofilm growth within the model system, but that it reached the highest densities at 65 °C (Table 3.2).

Within the variation observed, and as far as can be determined from the limited dataset, continuous inoculation and pulsed systems produced similar apparent growth rates for the range of temperatures tested. Similarly, observed times to development of new cells and spores were not affected greatly by applied flow rates on the system; whether 20 or 40 mL/min.

Fouling can be visibly detected with scanning electron microscopy, aided with electron-dense staining, but in this study biofilm densities were assessed by swabbing using cotton swabs, and viable count enumeration methods.

Moore and Griffith (2002) studied the efficacy of traditional swabbing

procedures and found that while 90% of the bacteria present on a stainless steel surface could be removed during swabbing, only ~10% of bacteria could be recovered and counted, due to problems of release of the cells and spores from the swab into the suspending medium.

The initial analyses suggested that generation times at temperatures from 60 to 67.5 °C, close to optimum for rapid growth, were in the range 24 to 37 min, similar to the results of others (Neilson et al., 1959; Ng and Schaffner, 1997; Flint and Brooks, 2001; Ng et al., 2002; Hetzer et al., 2006). The total volume of the flow-through chamber (~85 mL), and a milk flow rate of 20 mL/min means that the milk in the chamber is effectively replaced approximately every 5 min. Thus, the results (Table 3.1 and 3.2) implied that the increased cell and spore densities in the effluent arise from a growing biofilm, a fraction of which are released into the milk flowing through the chamber. Since the milk only resides in the chamber, on average, for less than ~ 5 min, planktonic growth in the milk flow cannot provide the amount of contamination observed in the effluent over the duration of the experiments. Thus, the spore loads must arise from the inoculated spores attaching to, germinating, growing and sporulating and ultimately dispersing from the surfaces within the flow-through system. The only estimates of cell and spore concentrations in biofilms within the chamber are from, the end of the runs. Nonetheless, if the total number of cells released into the effluent milk in the last hour of each of those experiments is estimated, these values can be related to the total number of cells attached to the internal surfaces of the chamber to provide approximate estimates of the rate of production of new cells and endospores by the

attached biomass, as was demonstrated.

Sporulation is a complex process, involving the coordinated actions of scores of genes, and has been reported to take 6 to 12 h in model species such as *B. subtilis* under otherwise optimal conditions (Parker et al., 1996). It was noted that the number of spores formed as a proportion of the total population was small, typically 1 in a 100 or 1 in 1000 cells. This suggests that extensive growth of the biofilm occurs before significant numbers of spores are released into the milk flow. It also suggests that sporulation is not a major activity for the biofilm associated cells, which is as expected given that the flowing milk provides nutrients, moisture and warmth and sporulation, and that sporulation is stimulated under nutrient stress conditions. Nonetheless, the rapidity of appearance of 'new' spores, suggests that sporulation may occur in only a few hours under near optimal conditions of temperature, water activity and nutrient availability as provided in these experiments.

3.4.1 Conclusions

The bench flow-through reactor system is a useful way to study the attachment, growth and sporulation of thermophilic-spore forming bacteria in a system simulating milk processing equipment and provides a tool to begin to develop interventions to reduce spore contamination of milk powders.

Studies using the bench flow through reactor showed that the number of *G. stearothermophilus* released into the effluent differed between the continuous flow spore-feeding experiments compared to the pulsed flow

experiments. This phenomenon is presumably due to greater numbers of attached *G. stearothermophilus* building up more quickly and being associated with a surface-fouling layer. The experiments performed are quite similar to that of Flint and Brooks (2001) but more information has been generated about the growth rate responses to a wider range of temperatures.

These data can be used as a foundation to estimate the expected benefits of changes to temperatures of operation of milk powder processes. It has also established a reliable experimental set-up to enable investigation of the kinetics of attachment, biofilm formation and development, outgrowth and spore-formation of thermophilic spore-forming bacteria. This system can be used further to conduct laboratory scale experiments to explore potential strategies to extend run times, for example utilising temperature cycling as foreshadowed by Knight et al. (2004).

4 The effect of temperature step changes on the growth and sporulation of *Geobacillus stearothermophilus* W14 in a model flow through reactor system

4.1 Introduction

As discussed in Chapter 1, *G. stearothermophilus* is a spore-forming bacterial species, which causes problems in the dairy industry due to its resistance to high temperature, including the pasteurisation process (72 °C, for 15 s) (Flint et al., 1997; Burgess et al., 2010). This thermophilic bacillus is able to attach to equipment surfaces throughout the milk powder manufacturing process, especially evaporators, and forms biofilms (Palmer et al., 2010; Marchand et al., 2012). Spore formation and sloughing of biofilms from surfaces can lead to end-product contamination (Stadhouders et al., 1982; Flint et al., 1997; Scott et al., 2007). Additionally, biofilm formation can also disrupt milk flow rates and accumulate in parts of milk powder plants that are hard to access for cleaning (Austin and Bergeron 1995; Flint et al. 1999; Hinton et al. 2003). Cleaning and sanitation in diary powder plants is typically required after milk powder is produced continuously for 18 to 20 h (Hinton et al., 2003; Bremer et al., 2006).

There have been numerous attempts to control and eliminate *Geobacillus* and related bacterial biofilms in milk powder industrial systems, including trialling different cleaning procedures (Bylund 1995; Bremer et al., 2006) and redesign

of equipment (Hamadi et al., 2014; Hotrum et al., 2015) however, the problem is intransigent. *G. stearothermophilus* typically grows from 40 to 70 °C in the production lines of dairy plants (Burgess et al., 2010; Chapter 2). Spores begin to be detected approximately 9 h into an 18 h manufacturing run (Scott et al., 2007) and increase significantly after 16 to 18 hours of operation (Scott et al., 2007; Chapter 3). Within their growth range, thermophilic bacilli display different physiological responses, especially during temperature shifts (Fields, 2001). Changes in lipid and fatty acid composition (Hasegawa et al., 1980), activity and stability of several enzymes (Lauwers and Heinen, 1983), protein stability (Wu and Welker, 1991; Weber and Marahiel, 2003; Beales, 2004; Tjalsma et al., 2004) and nutritional requirements (Souza and Martins, 2001) were reported. These different responses to temperature may provide a means to control biofilm formation.

There have been several attempts to control thermophile growth in processing lines using temperature cycling methods (Knight et al., 2004; Kaur, 2014; Zhao, 2014). Knight et al. (2004) conducted trials on the potential for temperature cycling to control the growth of *Streptococcus thermophilus* in milk pasteurisers in a pilot scale cheese-milk pasteuriser, where the temperature was shifted between 55 °C for 10 min and 35 °C for 60 min in the regeneration stage. These temperature shifts resulted in extending the time to detection of *S. thermophilus* to 20 h continuous production when previously they reached 10^6 CFU/mL in an 8 to 10 h run. Similarly, a temperature step-change study by Kaur (2014), using *G. stearothermophilus,* showed that the maximum extension of time to detectable contamination/reducing the

count was achieved when the temperature cycle was 55 ºC for 15 min then 35 ºC for 35 min. Notably, however, 35 °C is well below pasteurisation temperature but is also below the lowest temperature that permits *G. stearothermophilus* growth. However the most noteworthy and unusual results were that in initial experiments growth in controls was greater than growth in the step-change treatments. This was interpreted as the step-change, instead of restricting growth, encouraging the bacteria to attach more strongly to the coupons. It was noted that further trials to test this hypothesis were required (Zhao, 2014).

When *G. stearothermophilus* attaches to stainless steel, biofilms are formed, allowing subsequent multiplication and release of cells and spores into milk powders (Flint et al., 1997). The aim of the work described in this Chapter was to investigate the effect of different temperature cycles and flow rates on *G. stearothermophilus* W14 cell and spore formation and release from biofilms using a modification of the flow-through reactor system, previously described (Chapter 3). Cold milk was inoculated with *G. stearothermophilus* W14 spores and passed through two flow through reactors connected in series with the temperature shifted systematically within the range for *G. stearothermophilus* growth. Vegetative cells and spores were enumerated in the milk effluent at two points over time to determine the effect of step-change cycles on attachment, biofilm formation, and spore production and release. These results were then compared with the isothermal experiments performed in single chamber flow-through reactor system (see Chapter 3).

4.2 Materials and methods

Two custom-designed and built bench-top flow through chambers connected in series were used for all experiments, as shown in Figure 4.1. Two temperatures were applied and alternated every 72 min (60 min at constant temperature with an additional 12 min required to equilibrate to the new temperature), *i.e.*, so that each chamber oscillated between two temperatures, with opposite shifts between the two temperatures experienced in the two chambers over time. As in earlier studies at constant temperatures, milk leaving the system was collected at regular intervals and vegetative cells and endospores enumerated at approximately hourly intervals. The methods used for system inoculation and vegetative cell and endospore enumeration are described in Chapter 2 and 3.

Temperatures in each chamber were oscillated between:

i) 70 and 60 °C

ii) 63 and 50 °C

iii) 55 and 68 °C, and

iv) 55 and 45 °C.

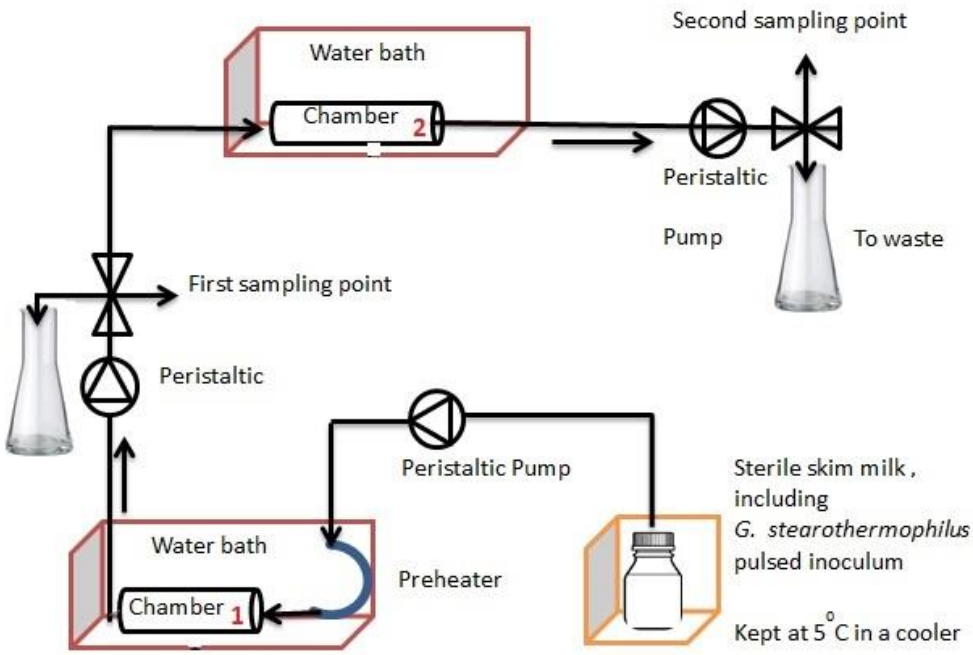


Figure 4. 1 A schematic showing the design of the dual flow-through experimental set-up to investigate the effects of temperature step changes on kinetics of *G. stearothermophilus* biofilm growth and spore and vegetative cell detachment in a stainless-steel flow through system.

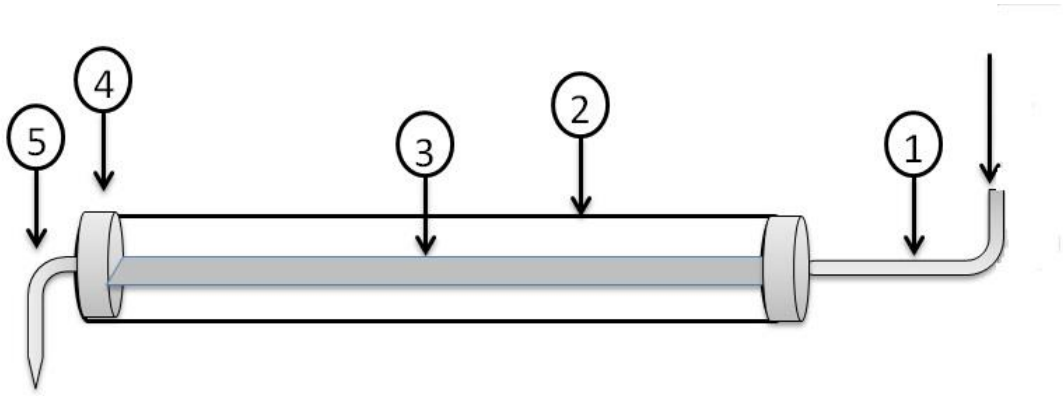


Figure 4. 2 A schematic showing the design of the chamber in a flow through system; 1--Inlet from milk reservoir and pre-heater, 2-Stainless steel chamber, 3-Stainless steel coupon, 4-Screw-on end caps, 5 Outlet and sample collection port.

Temperatures for the experiments were chosen to try to elucidate the effects of shifts from near optimal temperature for growth (60 °C) to super- and sub-optimal temperatures (70 and 60 °C, 63 and 50 °C), effects in the low temperature region for growth (55 and 45 °C), and temperatures equidistant around the optimal temperature for growth (55 and 68 °C). Growth responses of *G. stearothermophilus* W14 as a function of temperature in planktonic cultures were described in Chapter 2, and growth responses at constant temperatures in the flow through system were described in Chapter 3.

The underlying strategy in the experimental design is that at all times there was a zone of higher temperature, for each of the temperature pairs, but that the position of that region changed (i.e., by changing the temperatures in the two water baths). Thus, if *G. stearothermophilus* W14 required additional time to colonise the preferred temperature region alternately as temperatures shifted, a delay in response compared to a single region that was constantly at the higher temperature, might be expected. Accordingly, analysis of the effects of temperature shifts was attempted by comparing the attachment, growth and detachment kinetics observed in a single flow through chamber at similar incubation temperatures, to those observed in the modified, sequential, flow-through chambers subject to alternating temperatures.

G. stearothermophilus W14 spore suspensions, prepared as described in Chapter 2, were added as a pulsed inoculum into the flow-through reactor system at the nominated temperatures. Samples were taken from the effluent

every 5 min for the first hour and every hour thereafter for 24 h. Vegetative cells and spores were enumerated in samples of the milk effluent at the two sampling points (see Figure 4.1) using methods described in Section 3.2.4.

“Control” experiments were undertaken for growth/biofilm responses at constant temperatures of 60 °C, 65 °C and 67.5 °C and data generated as described above for time-dependent changes in numbers of vegetative cells released into the milk flow from biofilms as well as endospores.

4.3 Results

The kinetics of growth and spore production of *Geobacillus* spp over a wide range of growth temperatures using different growth media and incubation in Bioscreen C apparatus, water baths, and bioreactors were reported in Chapter 2 as well as those in a flow through reactor (see Chapter 3). Those data provide a reference data set for comparison of the results from the fluctuating temperature studies. A selection of growth temperatures, i.e. 70 to 60 °C, 63 to 50 °C, 55 to 68 °C, and 55 to 45 °C were chosen, for reasons described above, and to represent the temperature where thermophile growth could occur in a dairy powder plant.

4.3.1 Bacterial growth in a single flow through reactor

Figure 4.3 shows temporal changes in TVC and spore counts in the effluent under constant temperature conditions. As can be seen from Figure 4.3 there was a decrease in TVC number after the 1 h inoculum pulse with spore inoculum. Thereafter the TVC started to increase above the inoculum level

after 6 h especially at temperatures of 65 °C and 67.5 °C with TVC reaching ~10^8 CFU/mL after ~20 h. However, at 60 °C, with slightly lower spore inoculum level fed into the system, TVC started to increase above the inoculum level only after 16 h. This was surprising because 60 °C had been identified (see Chapter 2) as nearly the optimum temperature for growth rate. The results may have arisen because of the unusually low inoculum used for those experiments, suggesting that the rate of spore attachment, as influenced by spore concentration, milk flow rate and other conditions, may be an important element of the kinetics of biofilm development and new spore formation and release.

At 65 °C spore counts showed an increase above the inoculum level after 8 h and reached 5.5 log CFU/mL after 16 h. At 67.5 °C, the spore counts remained unchanged until 12 h at ~2 log CFU/mL then started to increase. As discussed above, for the 60 °C trial the starting inoculum was low, and new spore production was observed only after 20 h of milk flow. These data were intended for use as reference conditions for the temperature step experimental data but, unfortunately, the anomalously low inoculum for the 60 °C trials undermines comparisons to that data set. Despite the limitations, comparisons were attempted as far as possible.

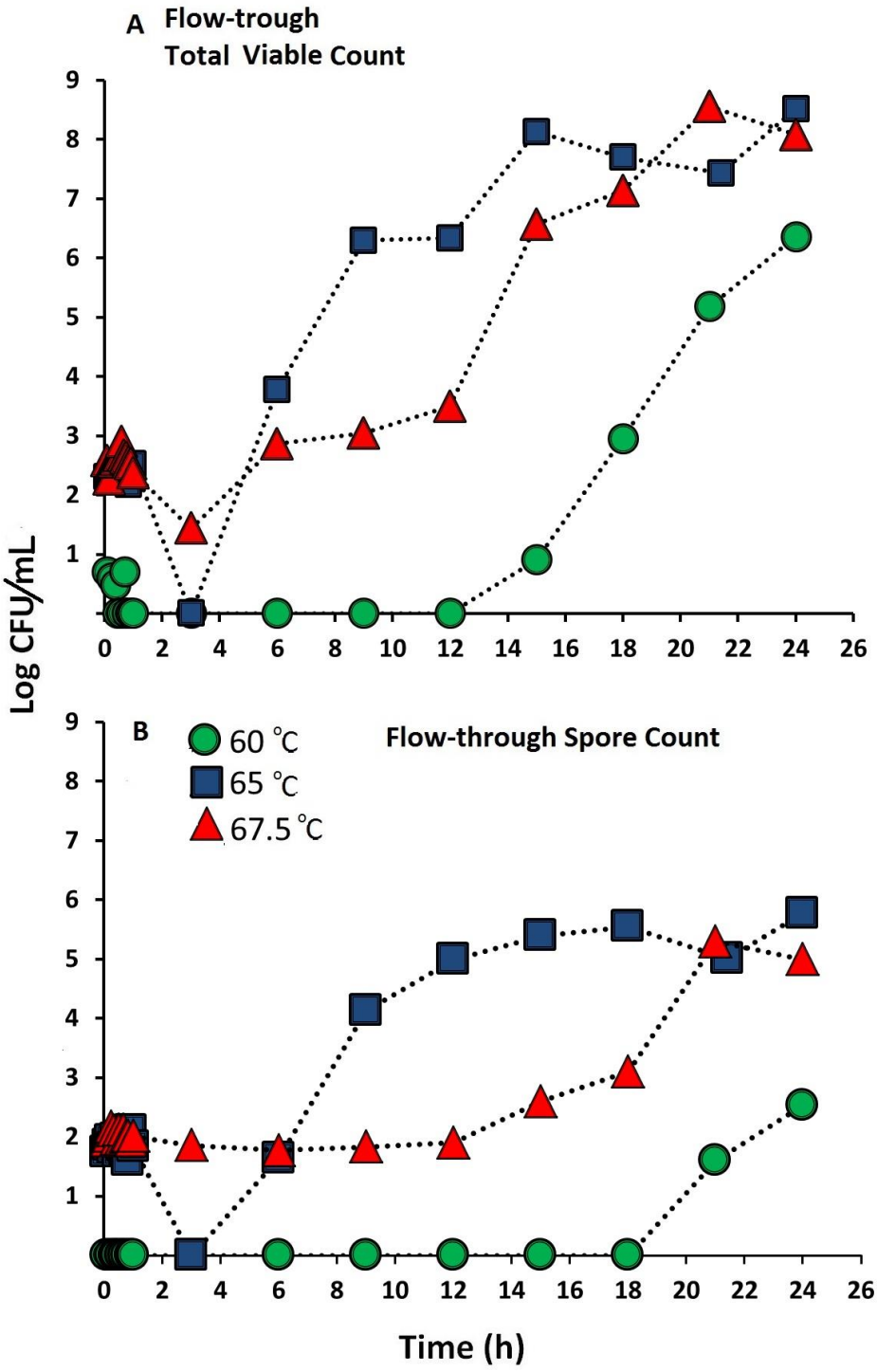


Figure 4. 3 Counts obtained on TSA plates for samples collected from the effluent in a flow through experiment (single chamber) during 24 h run at 60, 65, 67.5 °C. A) TVC; B) Spore counts.

4.3.2 Bacterial growth during implementation of temperature step changes

The implementation of temperature changes was conducted in four different temperature combinations, all within the growth-permitting temperature range for *G. stearothermophilus* W14 (see Chapter 2). Temperature step changes were 70 to 60 °C, 63 to 50 °C, 68 to 55 °C, and 55 to 45 °C, relevant to temperatures in dairy powder processing plants that are favourable for the growth *G. stearothermophilus*. The temperatures were alternated for 60 min for each set of temperatures. Twelve minutes were required to allow the water baths to adjust to the new 'set' temperatures every hour, i.e. so that a total of 72 min elapsed between withdrawal of consecutive samples.

Samples were drawn from both effluents periodically (72 min) to enumerate TVC and spore counts. Results (Figure 4.4 to 4.6) were compared to data from a single temperature flow-through system (shown in Figure 4.3) to attempt to assess the effect of temperature step changes. A 1 h inoculum pulse was applied for each trial and samples withdrawn every 5 min for the first 1 h, then approximately every 72 minutes afterwards.

At alternating temperatures of 70 to 60 °C (Figure 4.4), after a-1 h inoculum pulse, the TVC increased above the inoculum level after 8 h. The number was steady for ~2 h but started to increase to 10^6 CFU/mL. Spore counts showed no increase immediately after the 1 h spore inoculum pulse but started to increase above inoculum level after 10 h, increasing up to ~10^4 CFU/mL. The inoculum levels in this trial were, however, lower than in the fixed temperature

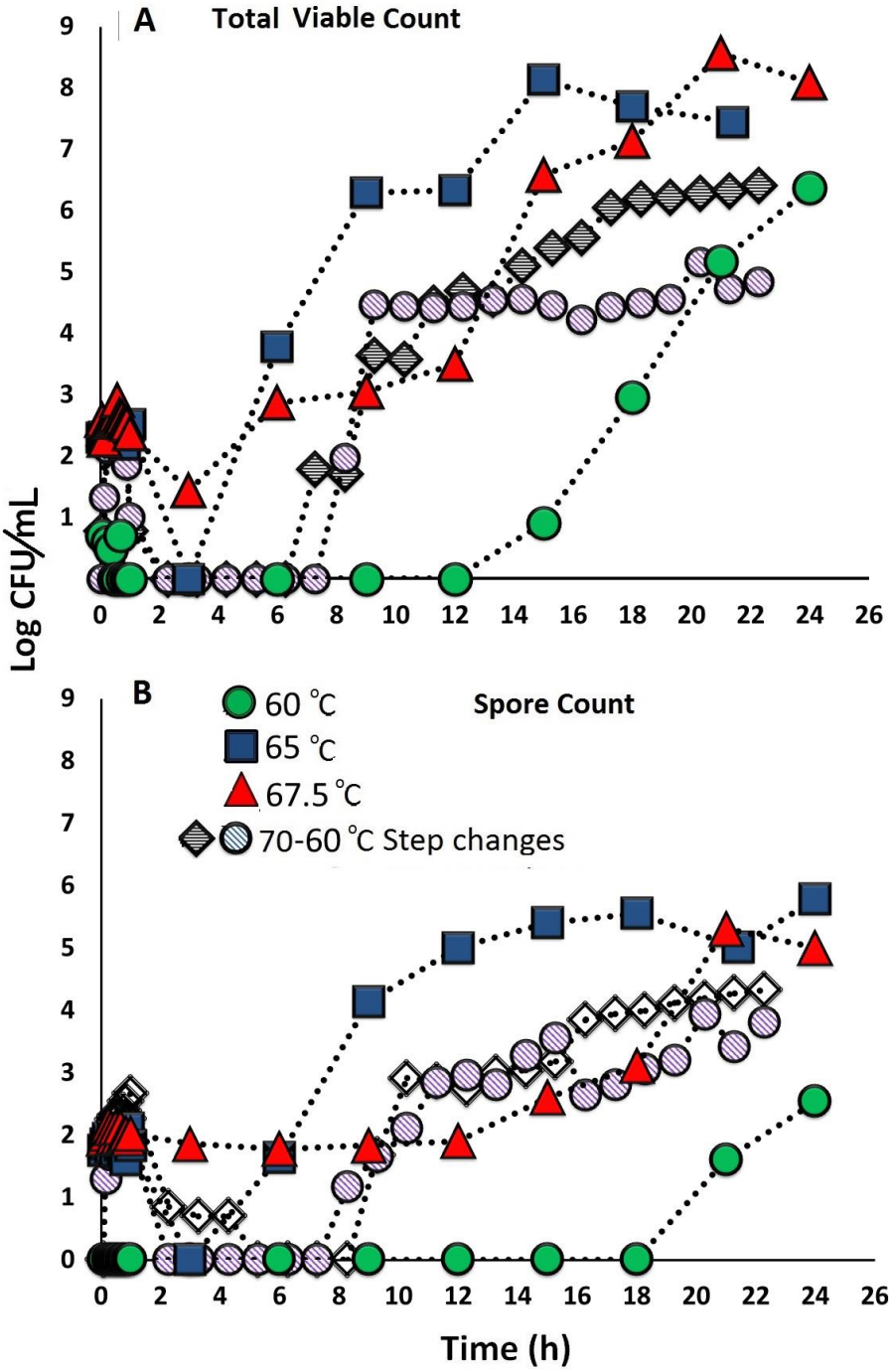


Figure 4. 4 TVC and spore counts over time in milk effluent during a 24 h experiment with temperatures in chambers alternating between 70 (grey diamonds) and 60 °C (lilac circles). A) TVC; B) Spore counts. Data are shown as *a function of chamber temperatures* and alternate between samples post chamber 1 or chamber 2.

control experiments (less than 10^2 CFU/mL), which may have extended times to detection of new cells and spores. Growth rates, however, would not be expected to be affected by inoculum density but appeared to be slower than growth rates in constant temperature experiments at 60, 65 or 67.5 °C.

Figure 4.5 shows that for temperature step changes between 50 and 63°C, TVC increased above the inoculum level only after ~10 h and that it reached a maximum 10^6 CFU/mL. Similarly, spore counts increased above the inoculum level only after ~10 h and reached a maximum level of 10^4 CFU/mL. The step changes did not retard growth compared to a constant temperature of 67.5°C, but did appear to delay growth compared to a constant temperature of 65°C.

Step changes between 55 and 68 °C (Figure 4.6) produced TVC increases above the inoculum level after 9 h and attained a maximum level in the effluent of 10^7 CFU/mL. Apparent growth rates under the fluctuating conditions were consistently lower than those of the constant temperature experiments at 60 or 65°C, even though the “average” temperature in the fluctuating condition experiments was 61.5 °C, i.e., very close to the optimum temperature for growth rate. Under the fluctuating conditions, a longer time (17 h) was required for spore numbers to increase above the inoculum level to achieve a maximum level of only 10^2 CFU/mL in the effluent. This result is also anomalous because of the very large difference between TVC and spore counts, being some 5 to 6 orders of magnitude. In all other flow-through experiments (see Table 3.1) differences between spore counts and TVC were typically only 2 to 3 orders of magnitude.

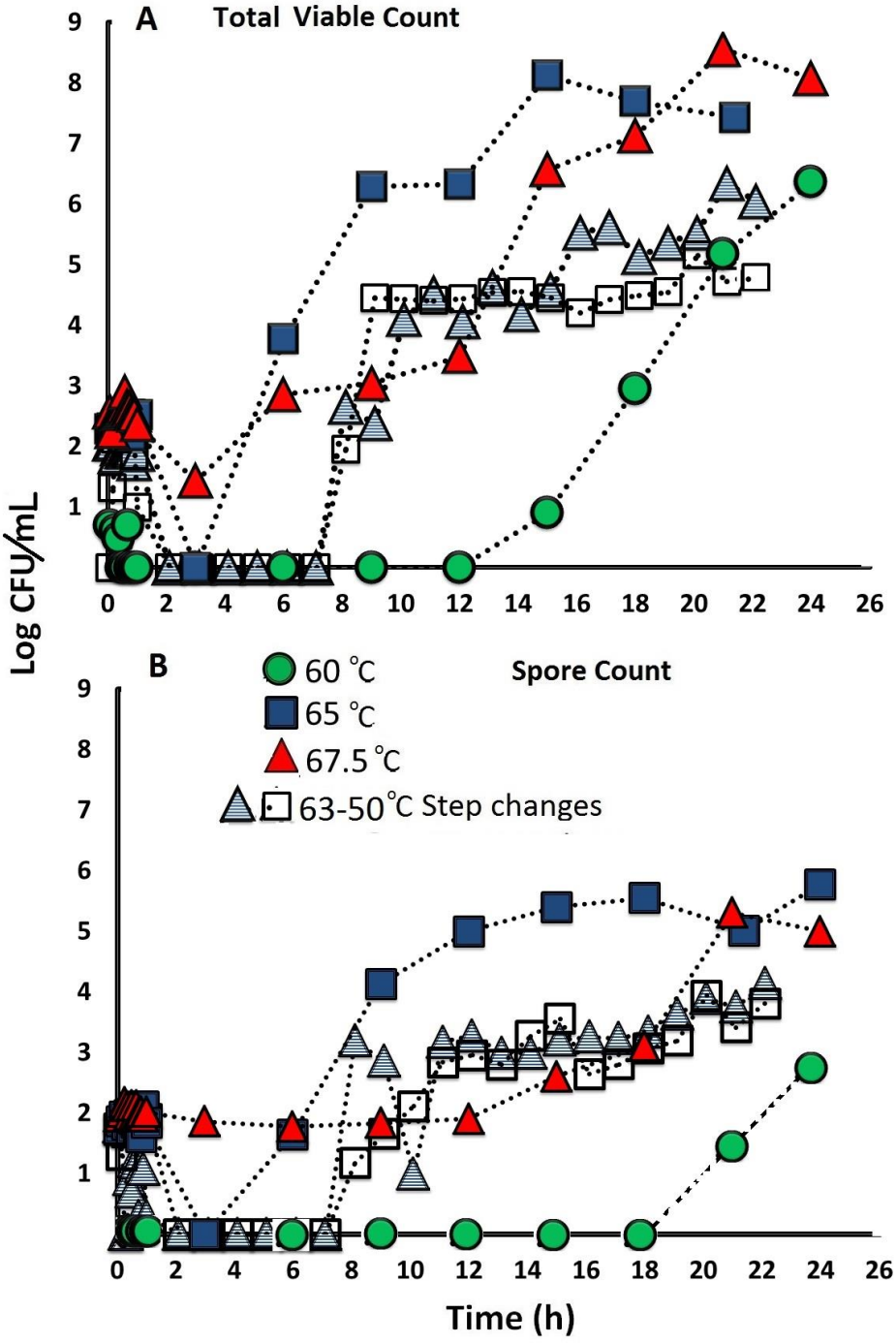


Figure 4.5 TVC and spore counts over time in milk effluent during a 24 h experiment with temperatures in chambers alternating between 63 (grey triangles) and 50 °C (open squares). A) TVC; B) Spore counts. Data are shown as *a function of chamber temperatures* and alternate between samples post chamber 1 or chamber 2.

At the lowest temperature combination involving oscillations between 55 and 45 °C (Figure 4.7), TVC numbers started to increase above the inoculum level after 12 h with the maximum level of 10^5 CFU/mL being reached after ~20 h, whereas spore counts did not show increase above the inoculum level throughout the 22 h duration of the experiment.

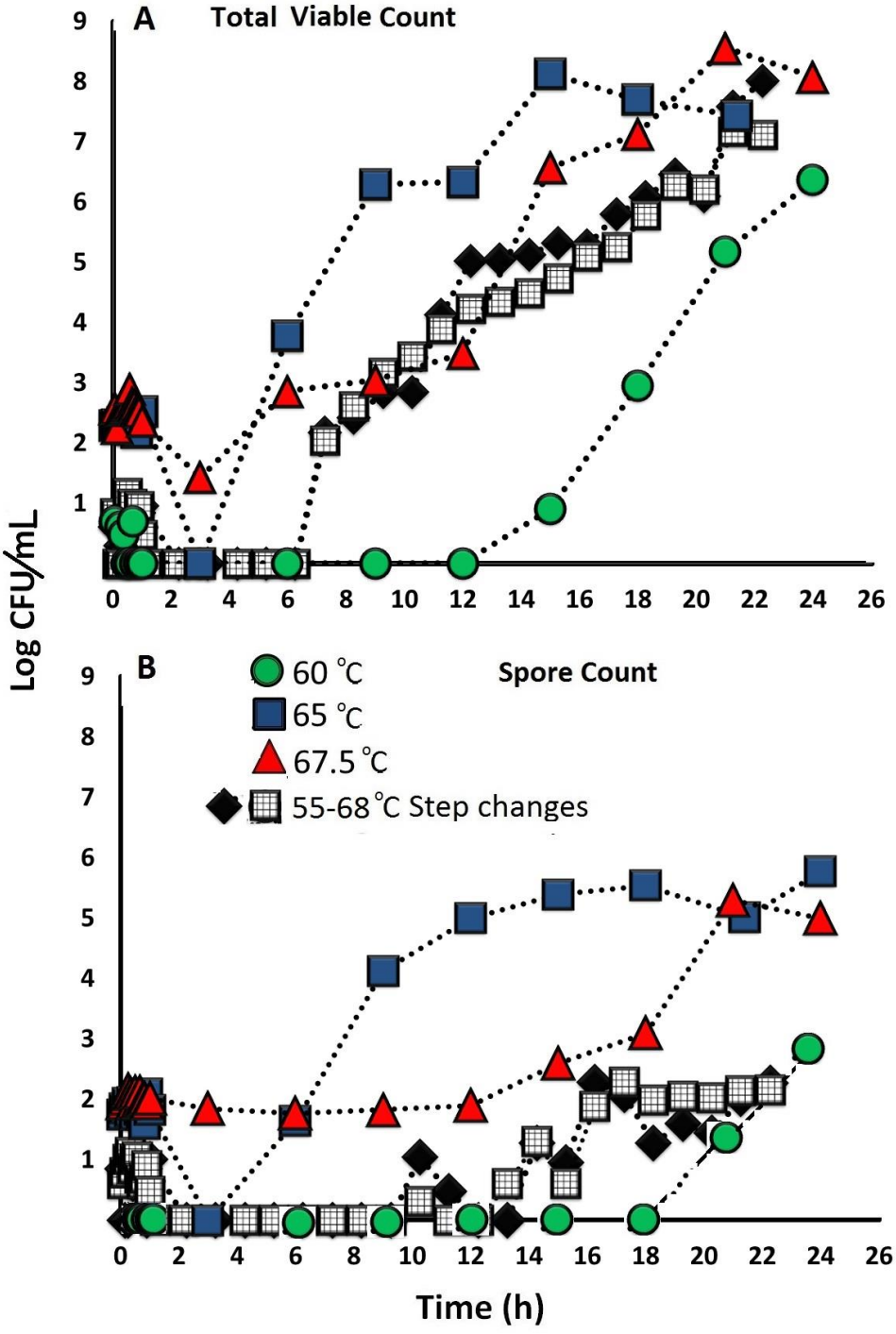


Figure 4. 6 TVC and spore counts over time in milk effluent during a 24 h experiment with temperatures in chambers alternating between 55 (black diamonds) and 68 °C (grey squares). A) TVC; B) Spore counts. Data are shown as *a function of chamber temperatures* and alternate between samples post chamber 1 or chamber 2.

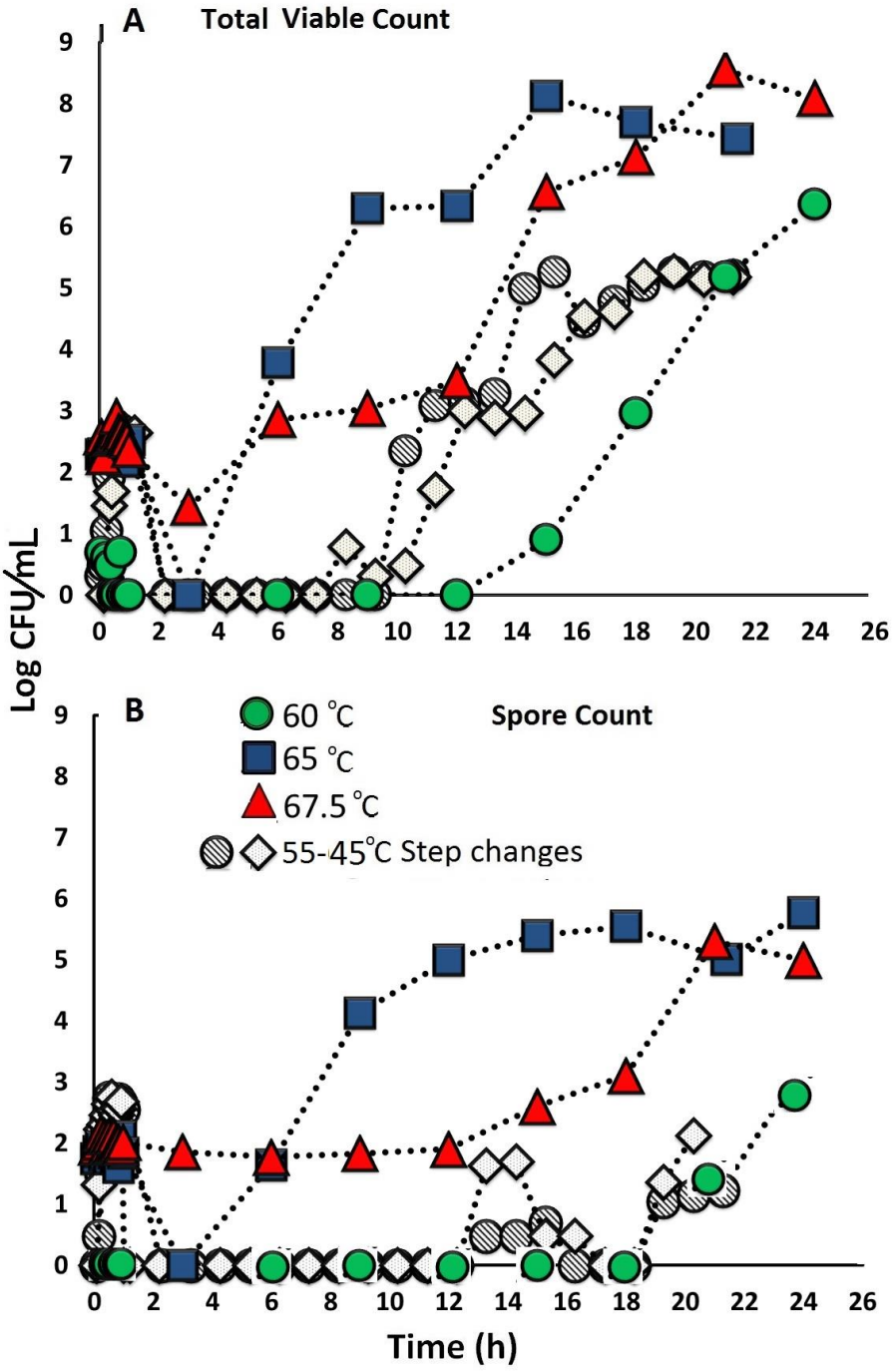


Figure 4. 7 TVC and spore counts over time in milk effluent during a 24 h experiment with temperatures in chambers alternating between 55 (grey circles) and 45 °C (open diamonds). A) TVC; B) Spore counts. Data are shown as *a function of chamber temperatures* and alternate between samples post chamber 1 or chamber 2.

The results of both spore and viable counts drawn from the effluent of the first and second chambers are shown in Figure 4.8. The graphs represent TVC and spore count numbers for all fluctuating temperature combinations of 70 to 60 °C, 63 to 50 °C, 55 to 68 °C, and 55 to 45 °C applied over a 24-hour “run”. They reveal whether there were any differences in kinetics in the first chamber and the second chamber. It was expected that counts in the effluent from chamber 2 would be somewhat higher than those from chamber 1, because the effluent from chamber 2 would contain cells and spores released from chamber 1 as well as those released from chamber 2. Alternatively, it might be expected that chamber 1 might start to release cells earlier than chamber 2, if most of the inoculum were captured by chamber 1, *i.e.,* reducing the spores available to colonise chamber 2. Neither effect is clearly indicated by the data available

In the second chamber, viable counts increased by 2 $\log_{10}$ CFU/mL more compared to the effluent from chamber 1 at the temperature combination of 63 and 50 °C; but showed a decrease in number during 70 and 60 °C step changes. The other temperature combinations appeared to show higher viable cells in the second chamber over time. Interestingly, the spore counts from chambers 1 and 2 showed no big differences in trends. Figure 4.8 shows that *G. stearothermophilus* W14 favoured temperatures between 60 to near 70 °C, although the microorganism was still able to grow well in the lower temperatures.

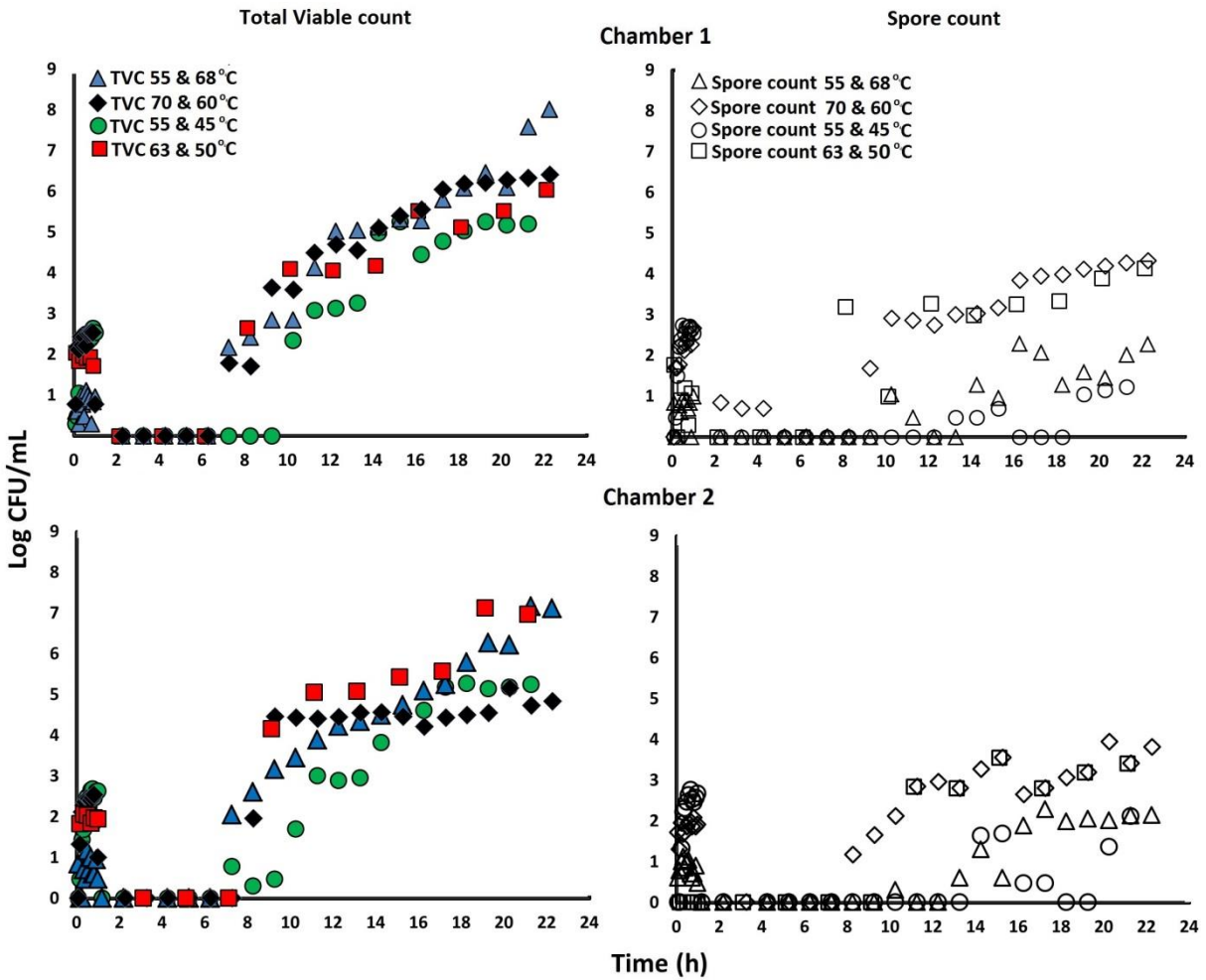


Figure 4. 8 TVC and spore count changes over time for samples collected from effluent from Chamber 1 or Chamber 2 sampling points (effluent) during a 24 h experiment with temperature in chambers 1 and 2 alternating between 55 and 68 °C, 70 and 60 °C, 55 and 45 °C, or 63 and 50 °C. Data are shown according to sample site.

It appeared that subjecting *G. stearothermophilus* W14 to temperature step changes within the growth range resulted in levels of TVC and spore counts lower than those using a single temperature in flow through reactor, and also led to slower (apparent) growth rates (data shown in Table 3.1). Also, in this study the time for spores and TVC to increase above the inoculum size was more than 9-hours within the optimum growth temperature range, which is slower than at analogous constant temperatures (see Table 3.1).

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4.4 Discussion

Unlike the studies of Kaur (2014), all the temperatures used for the step changes applied in this study were within the growth range of the test microorganism used, i.e., *G. stearothermophilus* W14. The complete data set for growth of *G. stearothermophilus* W14, including growth temperature, generation time generated using the flow-through reactor system is shown in Appendix 3. The range of temperatures used in these temperature step-change experiments represent both near-ideal conditions (~60 °C) for this microorganism to attach, develop into biofilms and slough off, as well as near growth limiting conditions (45 °C, 67.5 °C), and it is noticeable that the effect for oscillations involving 45 °C was much more pronounced than other temperature combinations. In fact, most other temperature combinations were not very ‘far’ away from the optimum growth temperature of ~60 °C (see Figure 4.8).

Although the growth of *G. stearothermophilus* W14 is affected greatly by different flow rates applied to the system (see Chapter 3), the temperature

step-changes seems to affect the growth rate, and onset of growth and biofilm formation, slightly. At the optimum temperature for growth rate of the thermophilic bacilli Flint et al. (2001) showed that *G. stearothermophilus* was able to form biofilms after 6 h incubation period using a laboratory continuous flow reactor, consistent with results presented in Chapter 3 which suggested that primary colonisation occurred within 3 to 6 h and that new spore production occurred after about 6 h. Others have observed that *G. stearothermophilus* can develop fully mature biofilms within 6 to 12 h (Costerton, 1984; Bester et al., 2010). Biofilms of *Geobacillus* spp can reach maximum cell density of approximately 6–7 $\log_{10}$ cells/cm^2 within 6 h (Flint et al., 2001; Parkar et al., 2003; Burgess et al., 2009).

The idea of implementing temperature step changes was to reduce *G. stearothermophilus* attachment and growth on the stainless surface of the flow through reactor by shifting the temperature every 1 h for 22 h, i.e., by interrupting or delaying the development of mature, densely populated, biofilms that could contribute high spore loads into the milk flow. It was proposed that this could be achieved by encouraging biofilm development to 'start again' at a new 'ideal' temperature location. While the results are not compelling, there is evidence that the temperature steps changes do slow the appearance of new cells and spores, may reduce growth overall (apparent) growth rate and may lead to lower spore levels in effluents.

Various approaches have been explored to reduce thermophile growth in dairy plants, including reducing the surface area available for biofilm

development, rapid increases in the temperature of the milk and, similar to the idea investigated in this Chapter, avoiding areas that provide the optimum growth temperature for thermophiles. Methods already applied to achieve this are dual preheating systems, direct steam injection (DSI), and indirect heating systems such as plate heat exchangers (Refstrup, 2000). In the dual preheating system, the milk is directed from one pre-heater to a second pre-heater after 8 to 12 h of processing.

As demonstrated by the results in this chapter, at a single temperature using a flow through reactor, the growth can lead to unacceptably high bacterial numbers in the product from 10^2 to 10^8 Log CFU/mL after 16 h continuous milk flow (see Chapter 3; Table 3.1).

Sporulation, under the influence of the temperature step changes at temperatures close to optimum for growth rate (see Table 3.1) was detected after 8 h of milk flow, whereas under constant single temperature conditions, sporulation was detected after 6 h. These findings are similar to Lindsay et al. (2005) who detected sporulation after 5 h in the planktonic culture using a flow through system compared to a static system.

As demonstrated in Chapters 2 and 3, temperature variation can have a considerable effect on the time to detectable growth and formation of spores by thermophilic bacilli, but the growth/sporulation rates have a broad temperature optimum (Chapter 2; Figure 2.7). However, as observed here (and described in Chapter 2), temperatures less than ~50°C can severely

retard spore formation, as has also been observed by Burgess et al. (2009) who compared spore production at 55 and 48 °C.

4.5 Conclusions

The data presented here and their interpretation is compromised by an inadequate experimental design (not all temperatures employed in the step changes had corresponding constant temperature controls) and anomalies in some of the 'control' data sets. Nonetheless, there is some evidence that temperature step changes might increase times to development of biofilms that release new cells and spores into the milk flow. It was apparent that, despite that one part of the system was always at one of the fixed temperatures, the apparent growth rates in the fluctuating temperature system were not as fast as that which would have been expected under the more optimal temperature of the step-change 'pair'. This possibility deserves more study and more rigorous experiments and sampling are required to reliably evaluate this possibility.

More detailed data analysis is also needed to determine whether the kinetics of biofilm formation in alternating temperature conditions are other than that which would be expected if the average of those temperatures were applied consistently throughout the trial. An intriguing observation, however, was that the yield of cells and endospores seemed to be lower in all fluctuating temperature conditions, than yields under static conditions of similar temperature. The data obtained also reinforced that at temperatures in the

range 55 to 65 °C there is little difference in growth rate of *G. stearothermophilus*, but that at 67.5 °C the detection of new growth took longer, suggesting that this latter temperature is above the optimum for growth, and causes growth inhibition. Accordingly, for temperature step changes to be effective, it would be expected that temperatures more extreme than those investigated in the work described in this Chapter might be required.

Knight et al. (2004) achieved localised temperature shifts of ~20 °C in a pasteurising operation in an effort to control *S. thermophilus.* More recently, Kaur (2014) concluded that step-changes from 55 to 35 °C for 35 min were effective in reducing *Geobacillus* biofilm growth rate and shedding into the effluent. The lower temperature in that study is outside the growth range and also outside the normal operating range of an evaporator, suggesting that it might not be technologically feasible in powder plants.

5. The Effect of Nitric Oxide on *Geobacillus stearothermophilus* W14 Biofilm Dispersal in a Flow through Reactor

5.1 Introduction

Chapters 3 and 4 of this thesis described the results of studies undertaken to explore thermophilic spore-former growth using the flow through reactor which mimics a dairy powder processing environment. This Chapter investigates the effectiveness of nitric oxide to disrupt *G. stearothermophilus* W14 biofilms within the flow through reactor. As suggested by Schreiber et al. (2011), the nitric oxide donor compound DETA-NONOate was hypothesised to disperse *G. stearothermophilus* biofilms on stainless steel surfaces.

As described earlier, biofilms are biological matrices produced by planktonic microorganisms that have typically attached to a surface, grown and become embedded within extracellular polymeric substances (EPS) that subsequently sloughs off at various times (Stadhouders et al., 1982; Flint et al., 1997; Sutherland, 2001; Stoodley et al. 2002, Scott et al., 2007).

Biofilms in dairy plants can cause milk fouling (Vyletùlová et al., 2000), which is of concern to dairy manufacturers since the fouling contaminates downstream products (Flint et al., 1997) as well as reduces heat transfer that can disrupt milk production (Kumar and Anand, 1998).

Within milk powder manufacturing plant equipment biofilm formation is influenced by five important factors: i) the species of thermophilic microorganisms attaching to the surface of stainless steel; ii) the actual manufacturing process; iii) the temperature applied during the process and time interval before cleaning takes place; iv) the grade and surface properties of the stainless steel used (Holah and Thorpe, 1990; Brooks and Flint, 2008); and v) the effectiveness of CIP procedures (Christensen et al., 1990; Morton et al., 1998). There are many methods proposed for biofilm prevention and removal from processing equipment, including a range of CIP systems (Bylund, 1995; Augustin et al., 2004; Bremer et al., 2006; Knight and Craven, 2010), as previously discussed. Nevertheless problems persist due to the need to manage the downtime required to perform these processes.

Novel strategies for removing biofilms exploiting bacterial systems that regulate biofilm formation and dispersal have been investigated (Beloin et al., 2014; Barraud et al., 2015). Although biofilm dispersal processes can be affected by changes in nutrient availability (Kaplan, 2010), or regulatory switches involving adaptation between oxic and anoxic states (Schreiber et al., 2011), it is also often linked to quorum sensing (QS). QS is a mechanism by which bacteria regulate gene expression on the basis of their population densities (Solano et al., 2014). In the case of biofilms, QS has been connected to the processes that result in the changes from cells transitioning from sessile to planktonic states (Shrout et al., 2011), i.e., dispersal from the biofilm.

Microbes in biofilms undergo developmental ‘programmes’, and cellular differentiation, resulting in a biofilm life cycle. One stage of this life-cycle is the induction and release of differentiated cells whose phenotype includes motility and other characteristics that enable those cells to escape from the EPS and to move to, settle in, and exploit other habitats.

The development of differentiated cells for dispersal is under the control of various environmental triggers and mediated by a variety of chemical signalling compounds produced by the microorganisms (Karatan and Watnick, 2009; McDougald et al., 2012; Oppenheimer-Shaanan et al., 2013; Zhao et al., 2013; Marvasi et al., 2015), including nitric oxide (NO). NO is used widely as a signalling molecule in biological systems including as a trigger for biofilm dispersal or detachment, e.g., by *P. aeruginosa* (Austin and Bergeron, 1995; Barraud et al., 2006; McDougald et al., 2012) interacting with many bacterial regulatory components, such as OxyR, SoxR, NsrR, NorR and regulators of the FNR family (Spiro, 2007). Several studies have suggested that exogenous NO can retard formation of biofilms, or encourage their disruption (Barraud et al., 2006; Barraud et al., 2009; Barnes et al., 2015; Lou et al., 2015; Marvasi et al., 2015; Cuttruzola and Frankenberg-Dinkel, 2016). Although QS might be directly affected by NO, there has not been any evidence that it influences biofilm formation, for example, by affecting cyclic-di-GMP signalling (Arora et al., 2015).

Bacterial responses towards NO can be affected greatly by other factors such as nutrient availability, temperature, exposure duration/intensity, and the

presence of other bacterial species (Privett et al., 2012). The concentration of NO depends on the gaseous and aqueous phase, reacting directly with oxygen, and has a relatively short half-life (Kelm, 1999). Therefore, to maintain a physiological concentration of NO in the laboratory, an NO donor, for example DETA-NONOate, is used to maintain the expected level. DETA-NONOate has a half-life of about 20 h, produces only NO and non-toxic diethylenetriamine (DETA). Its break-down is not affected by the presence of light, metals, thiols or cells (Feelisch, 1998; Yamamoto and Bing, 2000; Wang et al., 2002).

5.2 Materials and methods

The equipment configuration of the flow through reactor and preparation of media used in this study, milk suspensions, strain growth, and sampling method has described in previously (Sections 3.2 and 3.5, Figure 4.2).

5.2.1 Chemicals

The NO donor Diethylenetriamine (DETA)-NONOate (Cayman Chemicals, Ann Arbor, MI) was used. DETA-NONOate was dissolved in 10mM NaOH solution just before adding to the media (sterilised RSM). Milk containing (DETA)-NONOate was protected from light and kept no longer than 12 h at 0 °C.

The preparation of DETA-NONOate

Four different concentrations of DETA-NONOate were trialled in these experiments, *i.e.,* in flow through experiments: 0.01 mM, 0.1 mM, 0.5 mM;

and in static culture experiments: 0.5 mM and 1 mM.

The molecular weight of DETA-NONOate ($C_4H_{13}N_5O_2$) is 163.2 grams/mole. The DETA-NONOate was dissolved in 0.01 M sodium hydroxide; NaOH solution stabilised the NO donor. The mechanism behind this was that the high pH prevents releasing NO.

To make 1 M sodium hydroxide, 40 gm (1 mole) of NaOH was dissolved in 1 L of distilled water. 10 mL from the 1 M NaOH solution was added to 990 mL distilled water to give a final concentration of 0.01 M NaOH. Prior to use, NaOH was filtered sterilised using a syringe filter 0.2 µm (Millipore, Australia). To make up 0.1 M of DETA-NONOate (16.32 mg/mL), 10 mg of DETA-NONOate was dissolved in 0.6125 mL of (0.01M NaOH). From this concentration, the 0.01 mM, 0.1 mM, 0.5 mM, and 1 mM were prepared according to the desired volume used during the experiment.

For the ‘biofilm’ experiments, influent milk containing NO was run through the flow-through system for 1 hour pulses (at 20mL/min) requiring 1.2 L of RSM (reconstituted skim milk), hence for 0.5 mM, 97.92 mg of DETA-NONOate (16.32 mg/mL) was dissolved in 6.13 mL (0.01M NaOH) before added into 1.2 L of milk.

The static culture experiments required only ~61 mL of RSM and/or BHIB, therefore 10 mg of DETA-NONOate (16.32 mg/mL) was dissolved in 0.61 mL (0.01M NaOH) before being added into RSM/BHIB to give a final concentration of 1mM.

5.2.3 Biofilm experiments

Reconstituted skim milk (RSM; see Section 2.2.4), inoculated with 100 CFU/mL spore preparations of *G. stearothermophilus* W14, was pumped (at 20 mL/min) through the flow through reactor for ~1 hour in a temperature controlled water bath at 65 °C as previously described (see Chapter 3) to inoculate the chamber. Before experiments were started two sterile grade 304b stainless steel coupons (2cm × 2cm × 0.1cm) were inserted inside the chamber for subsequent scanning electron micrography of biofilms. The experiments were conducted with pulses of milk containing the following DETA-NONOate concentrations: 0.01 mM, 0.1 mM, and 0.5 mM. DETA-NONOate was applied in the RSM as a pulse for 1 h at a milk flow rate of 20 mL/min. The treatments were performed in three trials with the following time combinations: trial 1: 1h pulses containing 0.01 mM at 6 & 12 h within a 24 h run; trial 2: 1h pulses containing 0.1 mM at 6 & 18 h within a 24 h run; and trial 3; 1 h pulses containing 0.5 mM at 4.5 & 9 h within a 20 h, run.

5.2.4 Flask experiments

Experiments to assess the effect of (DETA)-NONOate were done in flask cultures to use less of the reagent, i.e., because the experiments did not require dosing of large volumes of milk as in the flow through experiments. The preparation and enumeration of viable cells during the experiment were conducted using RSM and BHIB. The inocula contained 100 CFU/mL vegetative cells of *G. stearothermophilus* W14 grown at 65 °C as previously described (see Section 2.2.6). Before the experiments started two sterile grade 304b stainless steel coupons (2cm × 2cm × 0.1cm) were placed in the

flasks. The experiments were conducted for 9 hours in temperature controlled waterbaths (65 °C) with shaking (80 oscillations/min). DETA-NONOate concentrations applied were: 0.1 mM, 1 mM as well as an untreated control containing no DETA-NONOate.

5.2.5 Microscopic imaging

The SS coupons were aseptically collected at the end of the incubations (whether flask or flow through experiments) and prepared for scanning electron microscopy as described by Nilsson (2010; see Appendix 4). The fixed, coated stainless steel coupons and coagulated milk were viewed (≥ 10 fields each) using field-emission-type scanning electron microscope (Hitachi SU-70 Ultra High Resolution Schottky SEM) at an acceleration voltage of 5 kV.

5.3 Results and discussion

The results of all experiments are presented below as graphs of vegetative cell counts and spore counts in the milk effluents collected from the outlet of the system over time. In the Figures, as appropriate, the times of application of the NO treatments are also shown. The figure legends detail the concentrations of NO used, and the times at which those treatments were applied. The treatment time for each pulse of NO was ~1 h. At the completion of each experiment, TVC and spore levels on surfaces within the flow through reactor were also assessed as counts per cm^2. These data are also presented graphically.

Scanning electron microscopy images are also presented for both flow-through and static culture experiments.

5.3.1 Morphological analysis of biofilms following NO treatments

In both flask and flow through experiments, the biofilm on SS coupons and presence of coagulated milk was compared using SEM. Biofilms forming on coupons in both the flask and flow-through reactor experiments were generally similar in appearance (Figure. 5.1 and 5.2). The cells associated with coagulated milk (Figure 5.3) seemed to be interconnected by fibre-like structures similar those observed on the surface attached biofilms (e.g. flow-through reactor biofilms).

The size and structure of biofilms formed under different experimental conditions, i.e., using flow through reactor or flask cultures showed slight differences in thickness and extent of clumping as illustrated in Figures below, although this was not investigated systematically over all the contaminated stainless steel surfaces, e.g., the attached cells were often located in small, dense clusters of cells for either method of treatment.

SEM visualisation indicated that for both milk fouling and surface-attached biofilms there were no systematic differences in relation to the DETA-NONOate concentration added. Flask culture-derived biofilms did not show an effect of NO treatment (1.0 vs 0.1 mM) despite constant exposure, see Figure 5.2) or compared to the ‘flow-through’ biofilms. The slight visual differences in biofilms formed under the different methods could be due to the

culture conditions and the continuous exposure to NO in the case of the flask or random differences between the time and site of attachment observed by the SEM process. e.g., due to different time periods since the commencement of attachment processes thus affecting density, extent and the overall structure.

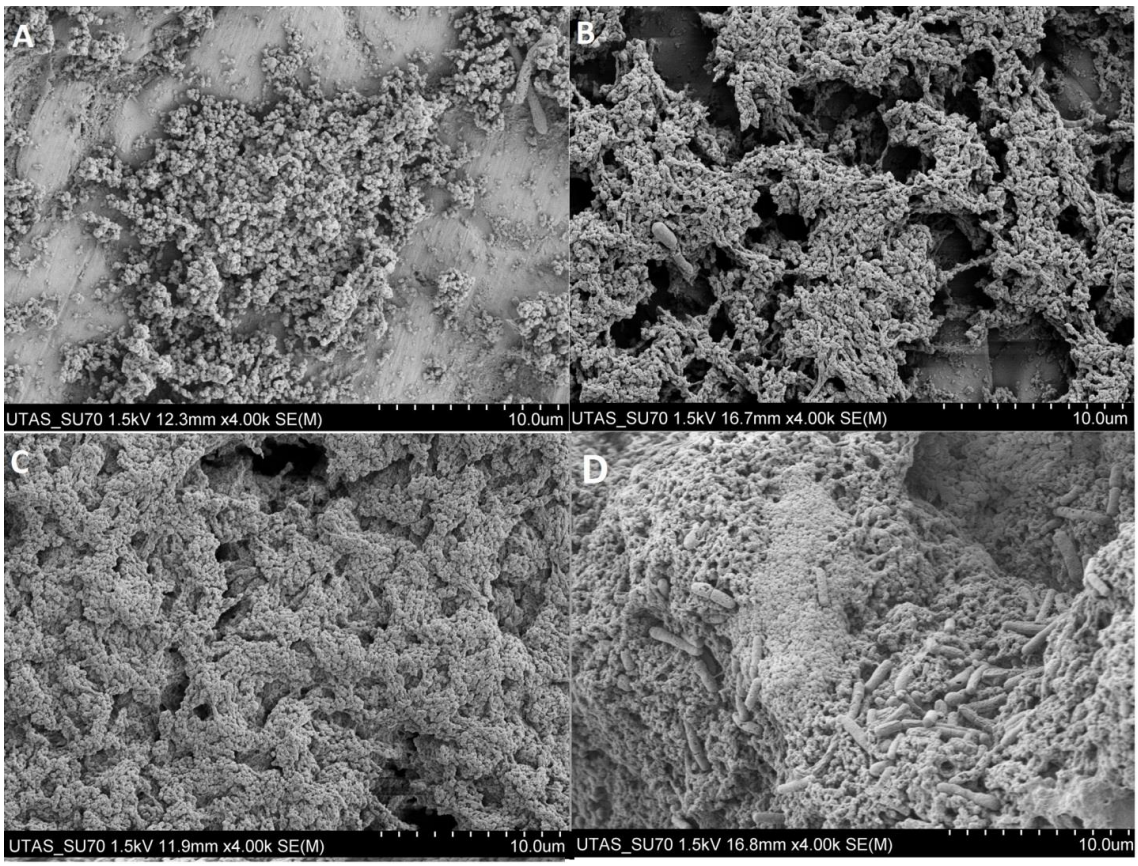

Figure 5. 1 Scanning electron micrographs of SS surfaces after exposure to (DETA)-NONOate treatments and time combination in the flow through reactor: A) 0.5 mM (4.5 & 9 h), B) 0.1 mM (6 & 18 h), C) 0.01 mM (6 & 12 h), D) untreated coupon (0 mM).

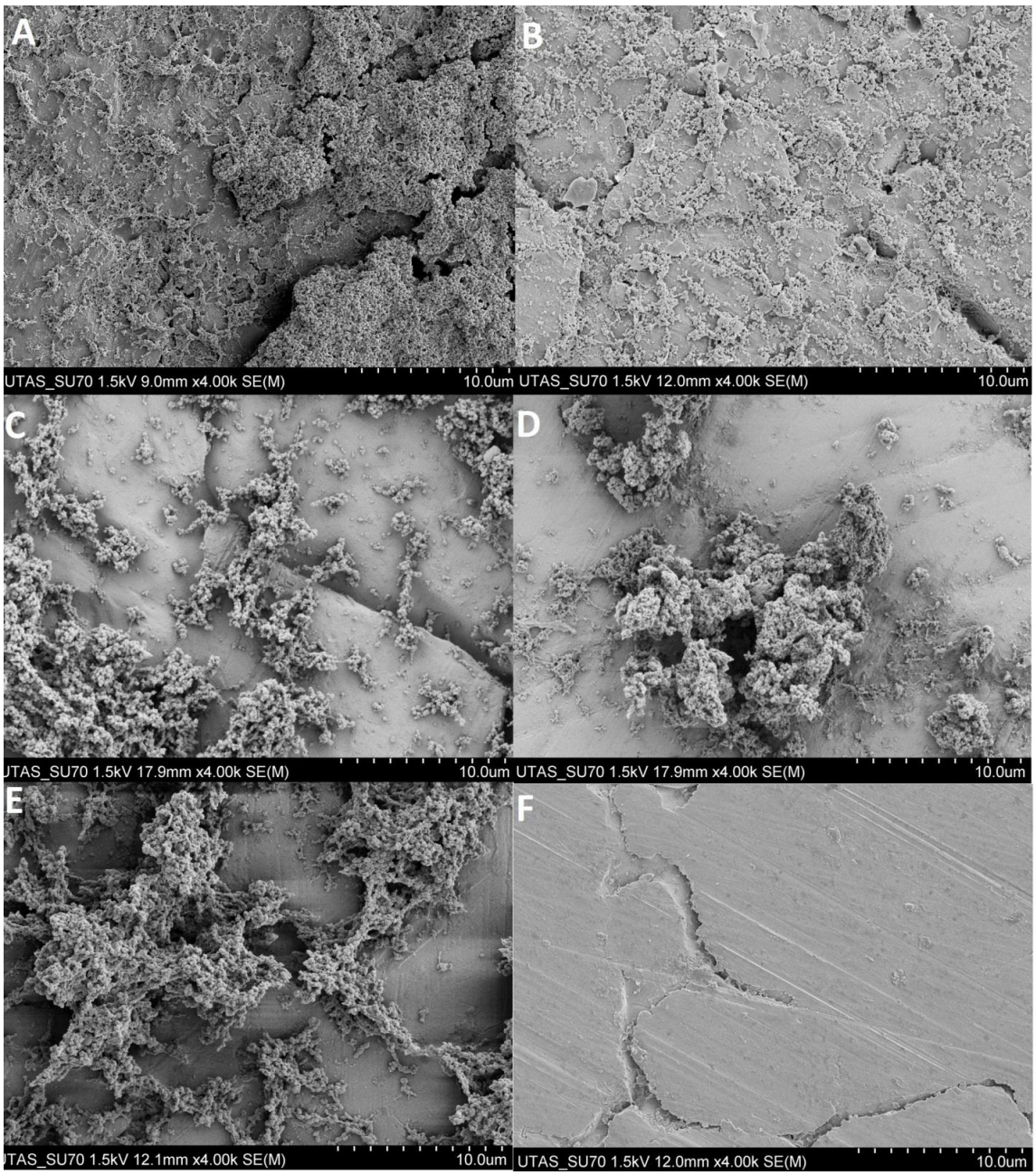

Figure 5. 2 Scanning electron micrographs of stainless steel surfaces after exposure to varied (DETA)-NONOate treatments in the flask (static) experiments at 65 °C for 15 h: BHIB A) 1 mM, B) 0.1 mM, RSM C) 1 mM, D) 0.1 mM, E) Untreated coupon (0 mM), F) coupon immersed in sterile RSM media.

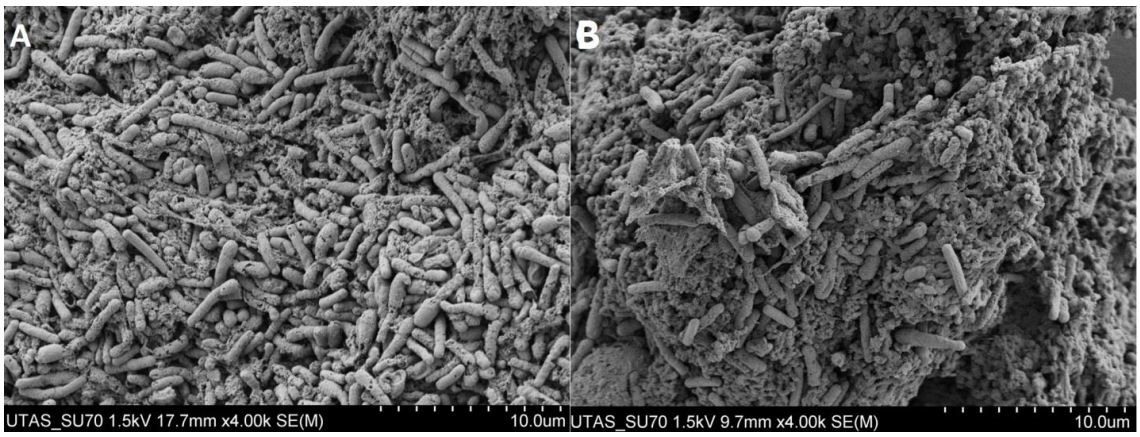

Figure 5. 3. Scanning electron micrographs of coagulated milk: A) control, B) after exposure to (DETA)-NONOate 0.1 mM in the flow through experiments at 65 °C for 20 h.

5.3.2 *G. stearothermophilus* W14 biofilm dispersal and associated tolerance to DETA-NONOate

To quantify the response to the DETA-NONOate treatment, the number of viable bacteria and spore counts in the milk effluent during 20 h of the trial were periodically determined. Various concentrations of DETA-NONOate were used, including much higher concentrations in flask culture compared to flow-through experiments and including levels that have been reported to disperse either growing or non-growing biofilms of pseudomonads (Barraud et al., 2015). The treatments applied, however, were found inadequate to disperse *G. stearothermophilus* biofilms in milk (Figures 5.4 and 5.5).

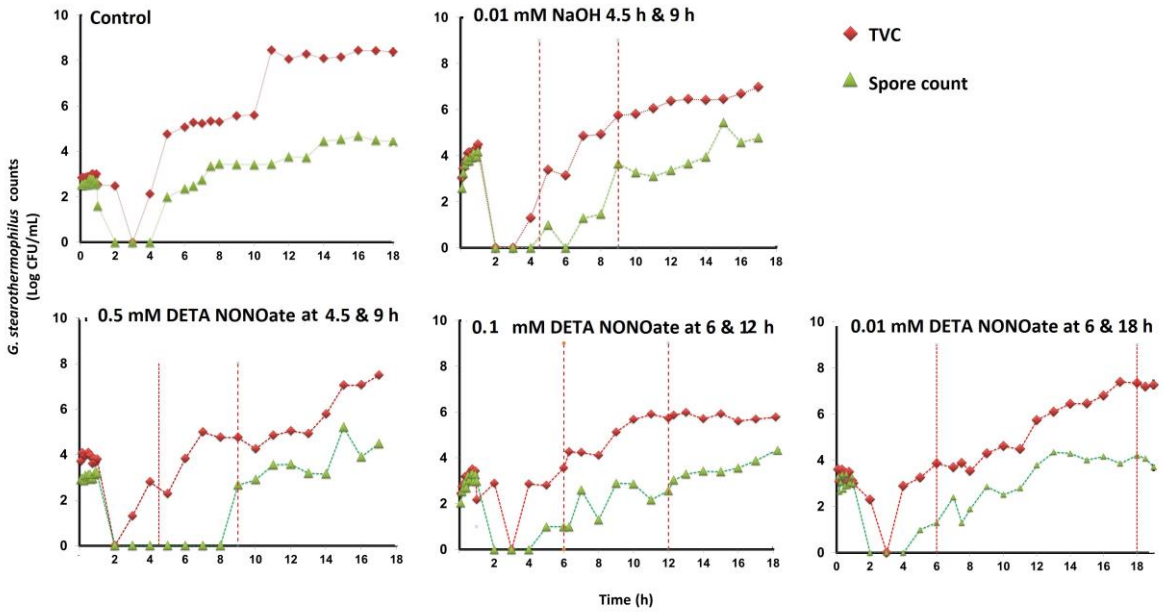

Figure 5. 4 (Upper). Changes in TVC and spore counts in milk that has passed through a flow reactor initially inoculated with a low level of *G. stearothermophilus* in milk for ~1 h, before being replaced by reconstituted skim milk without added *G. stearothermophilus*. This experiment represents a 'control', conducted at 65°C. (Right) Added NaOH (needed, in other experiments, to dissolve the DETA-NONOate) in all milk used in the trial. (Lower). Counts obtained from RSM broth inoculated with 100 CFU/mL *G. stearothermophilus* W14 spores and exposed to varied (DETA)-NONOate treatments in the flow through reactor. NO application times are indicated by the dashed lines.

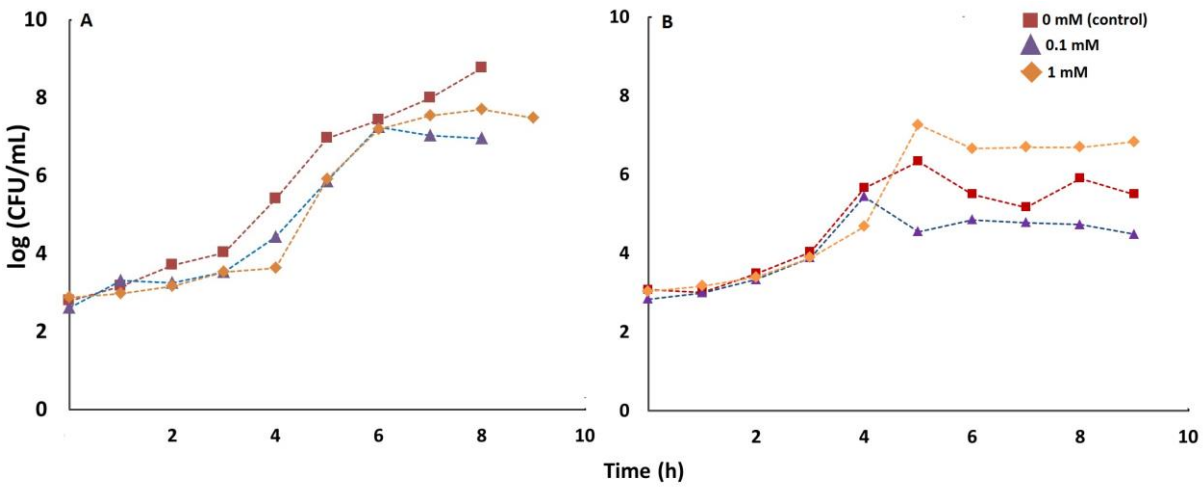

Figure 5. 5 Vegetative cell counts recovered on TSA over time from A) RSM, and B) BHIB inoculated with *G. stearothermophilus* W14 in flask (static culture) experiments at 65 °C for 10 h and exposed to 0.1 mM and 1 mM DETA-NONOate concentrations.

Responses represented in Figure 5.4 and Figure 5.5 suggest that there is no systematic effect on dispersal since, e.g., there was no evidence of any sudden increases in counts in the milk effluent coinciding with application of milk dosed with NO.

Figure 5.4 shows the growth of *G. stearothermophilus* W14 in the flow through reactor; using sequential sampling of total viable cell and spore counts every 1 h over 20 h. it also shows the growth of untreated controls and controls that include 0.01 mM NaOH (the solvent needed for solutions of DETA-NONOate. A *G. stearothermophilus* W14 spore inoculum was fed into the system during the first hour to assess the bacterial attachment (and potential detachment) during the run. Over the period of the assay, from hours

3 to 20, the microbial density in the effluent rose exponentially. Differences in the microbial density in the effluent over time, between DETA-NONOate and controls, were used as a measure of the effect of DETA-NONOate application at specified times.

G. stearothermophilus W14 cultures and flow-through experiments were treated with pulses of 0.5, 0.1, and 0.01 mM DETA-NONOate at various times. As seen in Figure 5.4, no systematic effects of DETA-NONOate on cell densities in milk effluents were observed. Log reduction counts in the effluent were suggested at hour 10, but the rate of regrowth was similar to that of the control. Importantly, if detachment had occurred, an increase in cells, or spores, in the effluent would have been expected rather than a decrease. In the presence of DETA-NONOate, generation time of the treatments were not systematically different for the 0.01, 0.1 mM and 0.5 mM concentrations (data are not shown).

Total viable cell and spore counts shown in Figure 5.4 and Figure 5.5 indicate that cell and spore numbers did not show consistent or systematic differences in response to the various NO treatments applied.

It was not possible to assess spores and vegetative cells on the internal surfaces during the experiment, since it was hard to open the chamber and to do so would have caused disruption to the milk flow. However internal surfaces of the chamber were swabbed at the end of experiment. A 1 log lower total viable count and 2 log reduction of spore counts for 0.5 mM NO

addition were observed compared with the control (Figure 5.6), though it was not possible to quantify the statistical significance of these apparent differences due to the difficulty and expense of conducting replicated experiments. However, enumeration of biofilm densities at the completion of trials (Figure 5.6) refutes this assessment, with no systematic differences in vegetative cell or endospore loads on different parts of the flow-through system, after treatments including DETA-NONOate, compared to treatments not involving DETA-NONOate.

Enumeration of biofilm densities on contact surfaces at the completion of flow-through trials (Figure 5.6) reinforces the above observations, with no systematic differences in vegetative cell or endospore loads on different parts of the flow-through system, after treatments including DETA-NONOate, compared to treatments not involving DETA-NONOate.

The response to NO could be complex in *G. stearothermophilus* since, like many bacilli, it possesses the means to generate NO via nitric oxide synthase (NOS) and reduce NO via nitric oxide reductases (H-NOX). NOS synthesises NO from L-arginine and oxygen (O_2) requiring NADPH (Kunst et al., 1997). Proposed functions for bacterial NOS proteins include protection against oxidative stress and metabolic nitration activities (Sudhamsu and Crane, 2009; Schreiber et al. 2011).

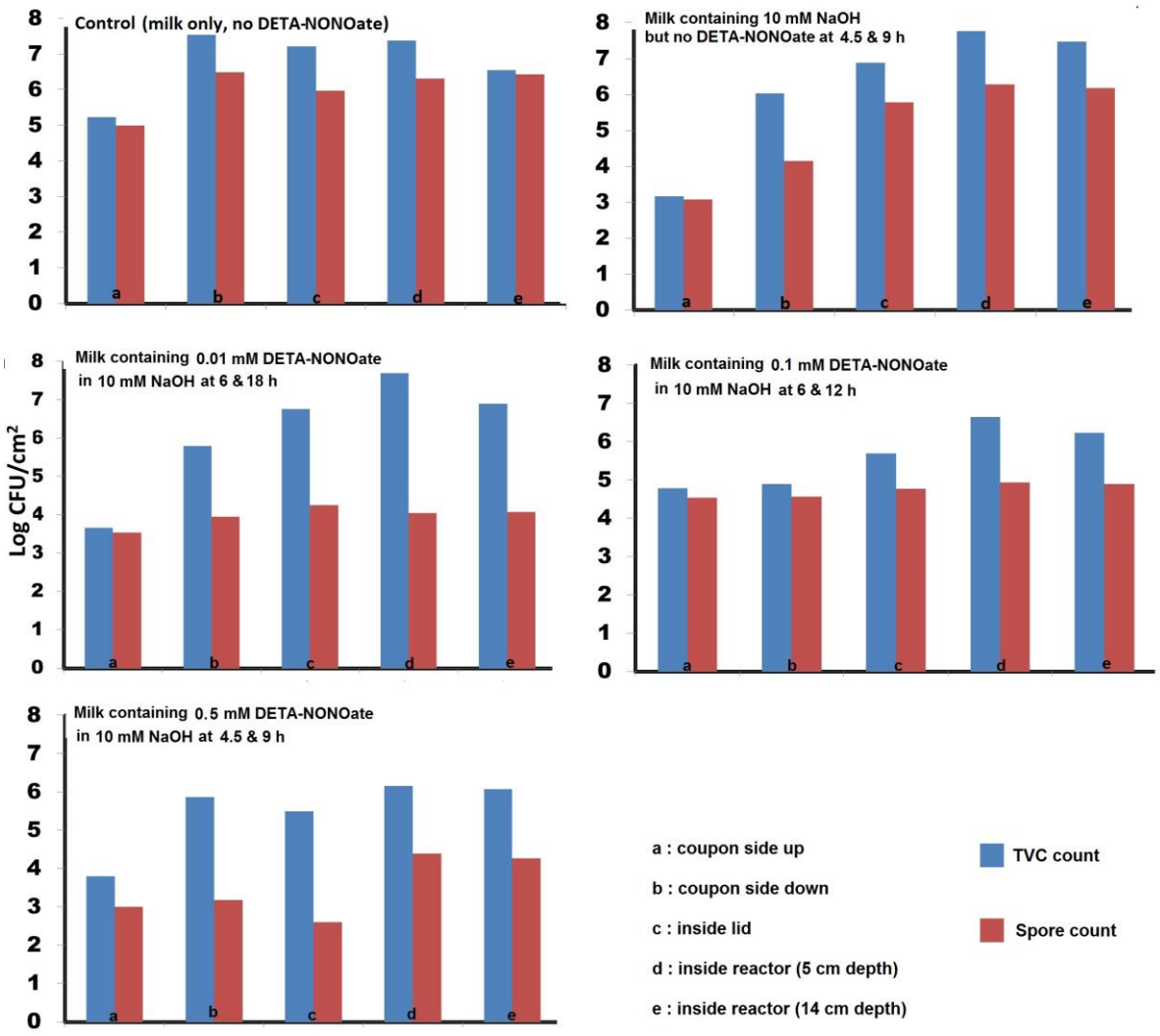

Figure 5. 6 Vegetative cell ('TVC') and spore densities (per cm^2) at various sites inside the flow through system. Milk containing various concentrations of DETA-NONOate in 10mM NaOH and control treatments (no additions, or NaOH only), was passed through the system after the standard pulsed *G. stearothermophilus* W14 inoculation procedure (~1 h), and 20 mL/min flow rate at 65 °C. Samples were taken after 20-24 h of milk flow.

H-NOX proteins seem to have a role in regulating bacterial biofilm formation by controlling NO levels or regulatory switches involving adaptation between

oxic and anoxic states (Schreiber et al. 2011; Derbyshire and Marletta, 2012) and influencing the physiological adaptations to anoxic conditions. H-NOX-converts NO to N_2O (nitrous oxide), which takes part in regulating anaerobic respiration of nitrate and nitrite (Rodionov et al., 2005; Lundberg et al., 2009). *G. stearothermophilus* also possess a NO-sensitive transcriptional repressor similar to that of *NsrR* in *Bacillus subtilis* that could act as a master regulator for the above responses including managing oxidative stress (Henares et al., 2013)

Triggers for dispersion of cells from a biofilm involve oxygen and/or nutrient limitations. Moreover, *G. stearothermophilus* W14 attached to stainless steel may be influenced by factors that allow this microorganism to survive within biofilms (Parkar et al., 2001). Although the mechanistic understanding of how NO effects biofilm formation by *Pseudomonas aeruginosa* (Barraud et al., 2006) is very preliminary, NO is known to be involved as a signalling molecule in biological systems. Thus its influence in signalling processes regulating biofilm formation could result in reduction of early biofilm formation.

Nitric oxide is a common chemical signal molecule in biological systems. It exists under standard conditions as a colourless gas. As such, it is not expected to leave residues in milk powder if it were proposed to use it to retard biofilm formation. DETA-NONOate is commonly used as a NO donor compound (Feelisch, 1998; Yamamoto and Bing, 2000; Wang et al., 2002). The exposure times the NO donor was applied in this study were compatible with typical milk production plant production times between cleaning. In this

study various doses of NO were used to attempt to induce dispersal of biofilm cells at (at least) one time during the development of the biofilm using the total vegetative cell count and the spore count to monitor biofilm dispersal into the milk flow, *i.e.*, assuming that more cells in the effluent suggests that more cells have attached, and also detached. A pulse at 4 to 6 h enabled assessment of whether the NO application induced dispersal, or may have delayed attachment. Since biofilm development, or delay of biofilm development, was not evident within four hours of seeding with cells of *G. stearothermophilus* W14, a second treatment was applied later in the process (e.g., at 9 to 10 h; see Figure 5.5).

Schreiber et al. (2011) investigated the role of NO in biofilm formation and dispersal of cells of a strain of *Bacillus subtilis* (3610) that exhibits swarming motility and develops complex biofilms. *B. subtilis* 3610 may represent an appropriate model system for exploring the role of NO dispersal of a Gram-positive, endospore-forming, bacterium. Zeigler (2014) reports, however, that the thermophilic spore-forming bacteria have considerably reduced genomes and proteomes compared to their mesophilic relatives. Nonetheless, the observation of Schreiber et al. (2011) provides insights into the apparent lack of effect of NO against *G. stearothermophilus* biofilms. Based on their rigorous investigations Schreiber et al. (2011) concluded that, rather than biofilm dispersal, endogenous NO derived from expression and activity of the enzyme nitric-oxide-synthase *accelerated* endospore formation and retention of cells in the biofilm. Furthermore, they noted that endospores are not dispersal cell phenotypes in *Bacillus* spp., but that motile vegetative

cells are the dispersal cell phenotype. They further reported that NO did not induce germination of spores, *i.e.*, that endospores were not precursors to dispersal cells. The role of spores is perhaps better understood as a bet-hedging phenomenon (Veening et al., 2008) under different sets of controls and evolutionary objectives.

Schreiber et al. (2011) concluded that "the effect of NO on dispersal is a species-specific phenomenon with different bacteria using NO for opposing dispersal strategies". They also observed that self-produced D-amino acids are known to insert into cell walls of Gram-positive biofilm bacteria and to disrupt the bonding between cells and the EPS, thereby promoting cell dispersal.

Taken together, the results from relevant literature suggest that exogenous NO is not likely to produce significant extension of powder plant run times. This is particularly true because the growth rates of thermophilic spore-formers under normal operating conditions in powder plants are rapid, with generation times at 65 °C in the range 15 -20 min (see Chapter 2). As such, as 50% reduction in cell numbers could only be expected to extend run times by 15 – 20 min, a 75% reduction might be expected to extend run times by about 45 min. To extend run-times by two hours would require reductions of biofilms load of 90 – 99%, far in excess of any reductions reported in the literature. Coupled with the costs of the reagents, and that the milk powder processing system is based on continuous flow of milk, including milk flowing past any developing biofilms in the system, it is difficult to envisage how

exogenous NO could be applied practically to extend run times.

5.4 Conclusions

These experiments were done to assess whether NO addition can induce *G. stearothermophilus* biofilm dispersal in industrial contexts. It was proposed that dispersal could contribute to control of biofilm growth, and thus use it as an aid for cleaning and the prevention of biofilms. The study indicated NO does not cause dispersal of *G. stearothermophilus* W14 biofilms based on TVC and SC numbers and also suggested by SEM imaging. Further research may be required to confirm that this is the case, but there is little evidence for it in the studies reported here or in the more recent published literature.

6. Final Discussion, Future Work, and Conclusion

6.1 Research benefits towards understanding spore-forming bacteria in Australian dairy products

During milk powder manufacture, the growth of thermophilic bacteria is usually influenced by the length of the process run time. In the case of long run times, e.g., 16 to 22 h, there is almost invariably an increase in thermophilic spore-forming bacterial contaminants in the end-product (milk powder) and, thus, increasingly lower customer acceptability. The level of contamination in the dairy powder plant determines the risk of contamination of end-products, and of consequent reduction of shelf-life of reconstituted milk powders, e.g., causing off-flavours. Thus, poor management of the milk powder manufacturing systems will usually lead to the manufacturer attracting customer dissatisfaction and economic loss.

With milk having a relatively short residence time throughout each stage of the dairy powder plant process, the most probable way for thermophiles to cause milk powder contamination is by forming biofilms (Scott et al., 2007). Biofilm formation on stainless steel surfaces within the plant equipment is associated with thermophilic bacterial attachment, growth including endospore production and, importantly, dispersal into the milk flow during milk powder manufacture. Biofilms are difficult to remove by cleaning because of the production of EPS which protects the cells from harsh environmental conditions and chemical exposure (Flint et al., 1997; Hinton et al., 2002).

Control of biofilms relies on the proper design of processing equipment to eliminate dead spores and niches, as well as correct implementation and application of cleaning and sanitising procedures (Parker et al., 2003). Because powder plants are large and complex machines, thorough cleaning is very difficult. Knowledge of locations in the process that are most likely to harbour and support thermophilic biofilm growth could aid in targeted cleaning strategies to minimise residual contamination and extend process run times.

This thesis was initiated to investigate the ecological responses of thermophilic spore-forming bacteria associated with dairy powder plants, in particular *G. stearothermophilus,* under various growth conditions and temperatures relevant to spore and biofilm formation and experienced during milk powder production, with the understanding that *Geobacillus* is a primary cause of milk powder contamination during manufacturing.

The research in this study focused on collecting data on the physiology and ecology of *Geobacillus* spp. isolated from various dairy plants in Australia. The collected data were developed and summarised into a predictive model to interpret the ecology of *Geobacillus* spp. by understanding vegetative cell growth and spore formation in relation to temperature and water activity. Further experiments were conducted to understand the germination, growth, and sporulation cycle of a representative *G. stearothermophilus* strain (W14). A quantitative predictive model for *G. stearothermophilus* growth rate as a function of temperature was developed that could be useful for improved management of spore issues within milk powder plants. Experiments were

also conducted to explore two proposed strategies to retard biofilm formation or assist in its detachment.

6.2 Approaches

The main purpose of this research was to generate new knowledge to identify strategies to minimise the number of spore-forming bacteria contaminating milk powders, with a view to extending plant run times and thereby increasing plant productivity. As suggested above, it was envisaged that this could be achieved through better management of processes, including identification of steps or locations in the processes where there is the greatest potential for thermophilic microbial contaminants to grow, and grow most rapidly, and subsequently contaminate end-products. The eco-physiological study of *Geobacillus* spp. was initiated by determining the upper and lower temperature and water activity limits for their growth, as well as the limits for spore germination and determination of growth rates focused on the representative strain *G. stearothermophilus* W14. These limits were tested in media with different compositions that were favourable for *Geobacillus* spp. growth. The information gathered from the growth behaviour suggested further experiments to explore ways to reduce the problem of build-up of spore-forming bacteria in dairy plant equipment.

Isolates used in this study were originally sourced from various Australian dairy powder plants by Dairy Innovation Australia Ltd. Species that were investigated closely were limited to *Geobacillus* spp. The identity of the strains was confirmed by 16S rRNA gene sequence analysis. The phylogenetic

analysis revealed the strains belonged predominantly to *Geobacillus stearothermophilus,* but included strains of *Geobacillus kaustophilus,* and *Geobacillus thermoleovorans* (>99% sequence similarity). All of these species have been reported to occur as milk powder contaminants in the dairy industry, in addition to *Anoxybacillus flavithermus* (Flint et al., 2009; Burgess et al., 2010). As a result, the subsequent modelling studies utilised strains that can be considered to have growth properties that allow them to become the dominant spore-forming thermophilic microbial contaminants.

Data obtained were used to develop a predictive model for *Geobacillus* spp. growth generated using various equipment, *i.e*., a Bioscreen C automated growth analyser, temperature controlled waterbaths, temperature gradient shaking incubators, bioreactors, and a purpose-built flow-through reactor. *Geobacillus* spp. growth was studied under various conditions to formulate descriptive mathematical models. A predictive square-root (Ratkowsky) model for growth rate was developed from the pooled data obtained from studies in broth media. On the basis of the 16 strains studied, the collective T_{opt} for growth was estimated as ~60 °C, while T_{min} and T_{max} were estimated empirically as 40 °C and 73 °C and fixed in the model fitting process. The range of temperature at which *Geobacillus* strains was observed to grow is somewhat narrower than the span of temperature for growth for many bacterial species (see e.g., Corkrey et al., 2012; Corkrey et al., 2014).

Growth does occur in many sections of the dairy powder process including

preheaters, evaporators, and plate heat exchangers. *Geobacillus* spp. biofilm formation occurs in sections of dairy plants that have temperatures ranging from 40 to 65 °C (Flint et al., 1997; Lewis, 2003; Scott et al., 2007), generally consistent with the growth limits of the *Geobacillus* strains estimated in this work and embodied in the predictive model developed. In some experiments (see Chapter 2), growth was observed at up to 70 °C depending on growth media, conditions and strain and growth at temperatures up to 67.5 °C was reproducible. Most of the *Geobacillus* spp. studied grew well on BHIB or RSM, but some strains grew poorly, or inconsistently, particularly in BHIB. *Geobacillus* spp. utilise carbohydrates, organic acids, peptone, tryptone, and yeast extract (Nazina et al., 2001; Marchant et al., 2006). *Geobacillus* spp. growing at high temperatures may need to first adapt their physiology to sustain growth at the new conditions: there are physiological changes required by *Geobacillus* for growth at high temperatures, such as the synthesis of phospholipids and fatty acid components (Nicolaus et al., 1995; Nazina et al., 2001; Romano et al., 2001) and these may induce specific nutritional requirements for the strains concerned.

From the 201 sets of growth data generated in this study it is clear that there is inherently high variability in growth rates, but that this does not appear to be systematically due to specific errors or deficiencies in the assessment of growth. The majority of strains used to generate growth rate data for the model exhibited seemingly erratic growth as measured by spectrophotometry, probably reflecting slight changes in the temperature of incubation and conditions. In a growth study of *Geobacillus* spp in a modified growth medium

(Jurado et al., 1987), erratic growth was observed above 65 °C. Changes in environmental conditions, such as temperature (including temperature shifts), pH, and a_w, during the growth phase may have resulted in these erratic growth curves, especially during the lag phase (Mellefont et al., 2003a, b; Le Marc et al., 2010). However, the bases of the apparently erratic growth of *Geobacillus* spp were not elucidated, and for this reason, it was difficult to develop criteria for inclusion/exclusion of specific data. As such, it was decided to develop the predictive model from pooled data for all strains, accepting the increased variance that this would impose on the model. However, the approach can also be defended because the model is then generic for *Geobacillus* spp. rather than potentially specific for a single strain, such as the recently presented model of Mtimet et al. (2015). However, other thermophilic genera may have different temperature growth ranges.

Although further determination of *Geobacillus* spp. growth rates under dynamic conditions, such as applying different flow rates and temperature cycling is probably necessary to be able to evaluate the reliability of the model, the model (generated using various growth media and wide range of growth temperatures) offers potential to predict the growth of *Geobacillus* spp. within dairy plants and to identify 'hot spots' for biofilm formation in dairy powder processes.

6.2.1 Characterisation of spore and vegetative cells attachment in dynamic conditions

Another goal of this work was to extend the base knowledge gained from

growth experiments in batch culture, by assessing the growth and spore formation of *Geobacillus* spp. when exposed to stainless steel surfaces held at temperatures typical of milk powder manufacturing equipment and with flowing milk. The kinetics of biofilm formation and maturation were also assessed, by measuring the number of vegetative cells and spores of *G. stearothermophilus* W14 in the effluent from a flow-through stainless steel reactor system during experiments extending over 22 to 24 h, and mimicking conditions in an evaporator in a dairy powder plant. The study was performed using temperature-controlled waterbaths at temperatures between 45 and 75 ºC. Although there was no consistent growth observed at temperatures above 67.5 ºC, as discussed above, this range of temperatures is found in various section of the dairy powder process and is conducive for *Geobacillus* spp. growth. The results of these studies, described in Chapters 2 and 3 of this thesis, provided useful insights about the kinetics of the attachment process and biofilm formation and the production of spores and vegetative cells of *G. stearothermophilus* under various constant milk flow rates and temperature conditions. *G. stearothermophilus* W14 was utilised since it exhibited representative growth responses for all sixteen strains of thermophilic spore forming bacteria investigated.

As mentioned above the measured growth rates of *Geobacillus* spp. were erratic and varied in different growth media and when using different enumeration systems. Growth was apparently slower than previously reported in the refereed international literature, particularly when using optical density methods, *i.e.*, a Bioscreen automatic growth analyser (static) and when using

BHIB as a growth media. In comparison, growth rates appeared faster in the flow-through system and particularly when the dilution effect was 'factored-in' to growth rate calculations, leading to generation time estimates at near optimal temperatures (60 – 65 °C) of 15 – 20 min, consistent with previous published reports.

Milk flow rate had no apparent effect on the time to development of biofilms or production (i.e., release into the milk effluent) of vegetative cells or spores. When the growth rate data derived from studies in the flow through system were compared to predictions from the model, it was found that the model provides good prediction of the growth rates observed in the pulsed-inoculum flow-through system.

At near optimal temperatures (~ 60 °C), the concentration of vegetative cells in the effluent flow for continuous-inoculum experiments started to increase after 1.5 h indicating that attachment, germination and proliferation occurs quite rapidly, whereas new spore development was detected only after 6 to 9 h. Overall, the results indicated the model system was a useful way to study the attachment, growth and sporulation of thermophilic spore forming bacteria in a system simulating milk powder processing equipment and that it provides a basis to develop interventions against spore contamination of milk powders. Swabbing different parts of the equipment at the completion of experimental "runs" showed various stages of biofilm formation within the model system.

Experiments using the flow-through reactor indicated that

G. stearothermophilus were released in the effluent earlier in experiments in which spores were added to the system continuously throughout the experiment compared to when the spores were added as single pulses of short (1 h) duration. The reason for the greater numbers is presumed to be due to more *G. stearothermophilus* attaching to stainless steel surfaces more quickly, forming biofilms and then releasing new cells and spores into the flowing milk earlier, because of the greater number of cells passed through the system. The experiments performed were similar to that of Flint and Brooks (2001) but, in this study, more information about the growth rate responses in relation to temperature was generated. The data can be used as a foundation to estimate the expected benefits of changes to temperatures in specific sections of milk powder processes. The work described has established a reliable experimental set-up to enable investigation of attachment, biofilm formation and development, spore-formation and outgrowth of thermophilic spore-forming bacteria. It can be used further to conduct laboratory scale experiments to explore potential strategies to extend run times, for example involving temperature cycling, or the potential of NO to disrupt biofilms early and extend plant run-times as discussed below.

6.2.2 Control of thermophilic growth

Bacterial spores are more resistant to externally applied stresses, such as heat and chemicals, than vegetative cells. The most common methods to control microbial contamination are by applying chemicals (disinfectants), or lethal temperature. Spores of *G. stearothermophilus* have very high heat resistance with D values at 120 °C of about 16.7 min (Davies et al., 1977) and

a mean z-value of 25.4 °C in the range 130-175 °C (Head et al., 2008). Vegetative cells of *G. stearothermophilus,* however, would be expected to become inactivated at temperatures >80 °C. As noted above, the generation time for *Geobacillus* spp. can be very rapid, estimated in these studies as ~15 – 20 min in the optimum temperature range 60 to 65 °C, consistent with observations of others. Consequently, the main thrust of this research was to explore ways to hinder spore formation, including hindering the initiation and growth of biofilms, rather than trying to hinder vegetative cells growth *per se*. Such a strategy was expected to have to be achievable without the need for major equipment redesign, and also to not require extensive use of chemical treatments to surfaces or in the milk liquid flow.

In this thesis (Chapter 4)*, G. stearothermophilus* W14 grown in sterilised skim milk was applied as spore inocula in milk passed through a flow through reactor system and temperature step changes imposed. The results (growth kinetics) were compared to analogous trials at isothermal conditions. This was done to determine if temperature fluctuations hinder growth and biofilm formation. At the optimum growth temperature (60 – 65 °C), the vegetative cell count increased after a 6 h “run time” reaching levels of 10^8 CFU/mL, whereas spore count increased after 8 h, reaching levels of 10^6 CFU/mL. The application of temperature step changes within the growth range of *G. stearothermophilus* W14 was found to delay the appearance of growth of vegetative *G. stearothermophilus* W14 cells and spores by 2 h. Under temperature step changes at near optimum temperatures, sporulation was detected after 8 h, whereas at constant temperature it was detected after only

6 h. Delaying the time of vegetative and spore development of thermophiles at optimum temperature presents an opportunity to powder manufacturers by allowing an increase in run times of milk powder plant before cleaning is again needed.

6.2.3 Nitric oxide application to disrupt biofilm attachment of *G. stearothermophilus* on stainless steel surfaces

G. stearothermophilus that are entrapped within biofilms are difficult to remove and show high resistance to disinfectants. Barraud et al. (2006), among others, reported the dispersal of biofilms using NO but noted that the effectiveness depends on the bacterial strain, temperature, and surface properties. In Chapter 5 of this thesis, studies on the effectiveness of NO, *via* a nitric oxide donor (DETA-NONOate) to disrupt *G. stearothermophilus* biofilms on the flow through reactor surfaces was described. The DETA-NONOate was added to milk at various concentrations within the time that biofilms were expected, from earlier studies, to start to grow; i.e. 4.5 h & 9 h, 6 h & 12 h, and 6 h & 18 h.

The studies of different DETA-NONOate concentrations applied at various times showed little difference in the apparent thickness of biofilms, as visualised by environmental scanning electron microscopy (ESEM). Similarly, there were no significant differences in vegetative cell counts and spore counts in the milk effluent, not even at times closest to the introduction of the NO donor compound. Minor perturbations in numbers in effluent milk and biofilm appearance were unlikely to lead to strategies to extend run times of

powder plants. The high cost of DETA-NONOate and the product availability make this method unlikely to be of benefit to the dairy for the industry. More significantly, more recent evidence suggests that NO does not act as a biofilm disruptor in Gram positive bacteria, such as *Geobacillus* reinforcing the observations in this thesis.

6.3 Future research

More research is needed to evaluate behaviour and growth responses of thermophilic spore-forming bacteria, other than *Geobacillus* spp. that can contaminate milk powder processing plants. In relation to spore germination and subsequent outgrowth, biofilm formation leads to contamination of milk flowing over the surfaces and leads to unacceptably high spore loads in powders over time. Other species may be expected to have different preferred temperature ranges for growth. If temperatures were altered in powder processing plants, would this simply create opportunities for other microorganisms better able to exploit those temperature ranges. Determining the minimum and upper temperatures for growth of other thermophilic spore-formers would help answer this question and the implementation of management options at dairy plants based on putative temperature manipulations.

Although the work presented in this thesis has yielded much useful information, the results also raised many further questions. For example:

i) Do physiological changes produced by biofilm growth enhance the

survival of bacteria in the milk powder process, enabling more to survive and sporulate?

ii) Are there any nutritional or physical factors relevant to milk powder manufacturing systems that can trigger spore germination within their growth temperature range

Areas of investigation specific to this thesis where more work is required include:

1) Further studies on the effects of flow rates and flow conditions (laminar or turbulent flow) to investigate their effect on cell adhesion to stainless steel surfaces, in comparison with previous adhesion studies (Sjollema et al., 1988; Sjollema and Busscher, 1990; Jaglic et al., 2011). The dynamic conditions in the flow through reactor may have an effect on the planktonic growth in the milk; e.g., the growth phase of biofilms can effect further cell attachment (Hood and Zottola, 1997). However, in the studies described in this thesis significant growth in the milk itself was discounted due to the flow rates used.
2) Investigations of nutritional and environmental factors, including temperature and water activity reduction, that may affect *Geobacillus* spp. spore formation processes occurring in the flow through reactor. This would improve understanding of the relevance of those influences in various sections of powder plants. Yeast extract, peptone, and glucose have been reported to contribute to spore yield as well as thermal resistance (Penna et al., 2003) and Ca^{2+} may also contribute to

signalling mechanisms (Herbaud et al., 1998; Ebrahimpour et al., 2008) that influence the degree of spore formation.

3) Developing better molecular understanding of spore germination and outgrowth is required for *Geobacillus* and related thermophilic species. This could be achieved potentially by transcriptomic and proteomic studies (Kimura, 2014). Constant heat challenge could result in development of increased resistance to heat and sanitiser chemicals (Austin and Bergeron, 1995), including cells in biofilms. Cells in biofilms are already protected by the polysaccharides or other EPS (Davies and Geesey, 1995). Although planktonic cells are thought to initiate interactions with a surface in response to various signals, including the nutritional status of the environment (O'Toole and Kolter, 1998; Pratt and Kolter, 1998), it is possible that quorum sensing plays a controlling role in a range of biological behaviours in cells that are detached from the biofilm and subsequently settle in new sites. Knowledge of mechanisms that influences quorum sensing within a biofilm community and gene expression is still limited (Parsek and Greenberg, 2005) is still limited for thermophilic spore formers
4) Calcium-binding proteins are known to be important for bacterial biofilm formation and may be involved in bacterial adhesion to surfaces (Craven and Williams, 1998; Latasa et al., 2006). Studying the effect of calcium variation in milk using the flow through reactor might offer insights that lead to a strategy to reduce adherence of cells, or spores, to stainless steel. Intercellular signaling (quorum sensing), has been

shown to be involved in biofilm development and might influence calcium-binding proteins in cells in biofilms (Vuong et al. 2003).

6.4 Final Conclusion

The objective of this PhD study was to improve understanding of the spore-forming thermophilic genus, *Geobacillus* spp. within in the context of milk powder plants. Milk pasteurisation practices are unable to eliminate thermophilic spores from entering milk powder plants, or from attaching and forming biofilms that lead to contamination in milk powders. The thesis has presented a description of the growth, biofilm formation and spore formation and release kinetics of *Geobacillus* spp. in response to temperature in a system closely mimicking elements of a powder processing plant, as well as preliminary information concerning the water activity responses of a representative strain of *G. stearothermophilus* (W14) that can further aid in identifying sites in an powder plant where the microorganisms could attach and proliferate.

The studies on biofilm formation, sporulation, and control in laboratory-scale flow-through reactors may be used to develop approaches to prevent the outgrowth of spore-forming bacteria during processing.

Additional putative strategies to control the growth of *Geobacillus* spp. such as temperature steps changes did not provide compelling evidence that this form of manipulation could substantially affect sporulation rates, but more rigorous and carefully planned and interpreted studies are warranted. There

was no consistent evidence of an effect of NO and, in fact, the available literature suggests that NO is unlikely to be successful as a biofilm disruptor in *Bacillus* or *Geobacillus* spp. Although quorum sensing was not specifically studied, information about cell stress, colony density, cell state and signals from other cells should be investigated further. Understanding and exploitation of such phenomena, that could delay or prevent biofilm formation, could lead to reduced levels of spores in the final product, the possibility of extended run times for dairy powder plants, and increased industry productivity as well as advancing scientific knowledge of triggers for sporulation and germination in spore-forming bacteria. Moreover, strain variability in the growth rate of a microorganism may be important knowledge for food safety (Aryani et al., 2015a, b), or food quality, management.

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APPENDIX 1. Complete dataset used for development of Equation 5

Temp (°C)	Strain	Gen. Time (min.)	Growth Medium	Culture System	Method Used
45	W5A	255.23	BHIB*	Bioscreen C	calibrated OD
45	W14	456.61	BHIB*	Bioscreen C	calibrated OD
45	W5B	431.57	BHIB*	Bioscreen C	calibrated OD
45	W7E	401.17	BHIB*	Bioscreen C	calibrated OD
45	T1C	1366.27	BHIB*	Bioscreen C	calibrated OD
45	103A	676.35	BHIB*	Bioscreen C	calibrated OD
45	W7D	311.99	BHIB*	Bioscreen C	calibrated OD
45	3D	1261.15	BHIB*	Bioscreen C	calibrated OD
45	291	1412.49	BHIB*	Bioscreen C	calibrated OD
45	T17	383.09	BHIB*	Bioscreen C	calibrated OD
45	W5D	181.70	BHIB*	Bioscreen C	calibrated OD
45	126	270.78	BHIB*	Bioscreen C	calibrated OD
45	W16	390.75	BHIB*	Bioscreen C	calibrated OD
45	28S	633.00	BHIB*	Waterbaths	viable count
45.5	W5A	105.62	BHIB*	Bioscreen C	calibrated OD
45.5	W14	120.25	BHIB*	Bioscreen C	calibrated OD
45.5	W5B	105.37	BHIB*	Bioscreen C	calibrated OD
45.5	W7E	131.51	BHIB*	Bioscreen C	calibrated OD
45.5	T1C	176.53	BHIB*	Bioscreen C	calibrated OD
45.5	103A	143.80	BHIB*	Bioscreen C	calibrated OD
45.5	W7D	111.68	BHIB*	Bioscreen C	calibrated OD
45.5	3D	133.82	BHIB*	Bioscreen C	calibrated OD
45.5	291	102.91	BHIB*	Bioscreen C	calibrated OD
45.5	T17	101.05	BHIB*	Bioscreen C	calibrated OD
45.5	W5D	98.42	BHIB*	Bioscreen C	calibrated OD
45.5	126	105.53	BHIB*	Bioscreen C	calibrated OD
45.5	W16	135.42	BHIB*	Bioscreen C	calibrated OD

Temp (°C)	Strain	Gen. Time (Min.)	Growth Medium	Culture System	Method Used
47.5	50B	122.11	BHIB*	Bioscreen C	calibrated OD
47.5	W5A	77.25	BHIB*	Bioscreen C	calibrated OD
47.5	W14	74.83	BHIB*	Bioscreen C	calibrated OD
47.5	W5B	81.57	BHIB*	Bioscreen C	calibrated OD
47.5	W7E	72.42	BHIB*	Bioscreen C	calibrated OD
47.5	T1C	100.74	BHIB*	Bioscreen C	calibrated OD
47.5	103A	89.11	BHIB*	Bioscreen C	calibrated OD
47.5	W7D	100.61	BHIB*	Bioscreen C	calibrated OD
47.5	3D	193.45	BHIB*	Bioscreen C	calibrated OD
47.5	291	70.07	BHIB*	Bioscreen C	calibrated OD
47.5	T17	79.04	BHIB*	Bioscreen C	calibrated OD
47.5	W5D	75.20	BHIB*	Bioscreen C	calibrated OD
47.5	126	68.13	BHIB*	Bioscreen C	calibrated OD
47.5	W16	101.43	BHIB*	Bioscreen C	calibrated OD
49	W14	750.60	BHIB*	Waterbaths	viable count
50.5	50B	152.63	BHIB*	Bioscreen C	calibrated OD
50.5	W5A	79.88	BHIB*	Bioscreen C	calibrated OD
50.5	W14	64.43	BHIB*	Bioscreen C	calibrated OD
50.5	W5B	51.93	BHIB*	Bioscreen C	calibrated OD
50.5	W7E	65.36	BHIB*	Bioscreen C	calibrated OD
50.5	T1C	76.59	BHIB*	Bioscreen C	calibrated OD
50.5	103A	54.68	BHIB*	Bioscreen C	calibrated OD
50.5	W7D	90.89	BHIB*	Bioscreen C	calibrated OD
50.5	3D	257.30	BHIB*	Bioscreen C	calibrated OD
50.5	291	63.41	BHIB*	Bioscreen C	calibrated OD
50.5	T17	73.46	BHIB*	Bioscreen C	calibrated OD
50.5	W5D	59.17	BHIB*	Bioscreen C	calibrated OD
50.5	126	84.83	BHIB*	Bioscreen C	calibrated OD
50.5	W16	84.55	BHIB*	Bioscreen C	calibrated OD

Temp (°C)	Strain	Gen. Time (Min.)	Growth Medium	Culture System	Method Used
51	W16	90.00	BHIB*	Waterbaths	Double dilution
51	126	60.00	BHIB*	Waterbaths	Double dilution
52.5	50B	104.50	BHIB*	Bioscreen C	calibrated OD
52.5	W5A	54.33	BHIB*	Bioscreen C	calibrated OD
52.5	W14	60.34	BHIB*	Bioscreen C	calibrated OD
52.5	W5B	48.84	BHIB*	Bioscreen C	calibrated OD
52.5	W7E	44.10	BHIB*	Bioscreen C	calibrated OD
52.5	T1C	54.32	BHIB*	Bioscreen C	calibrated OD
52.5	103A	52.77	BHIB*	Bioscreen C	calibrated OD
52.5	W7D	65.32	BHIB*	Bioscreen C	calibrated OD
52.5	3D	71.47	BHIB*	Bioscreen C	calibrated OD
52.5	291	42.74	BHIB*	Bioscreen C	calibrated OD
52.5	T17	50.36	BHIB*	Bioscreen C	calibrated OD
52.5	W5D	52.45	BHIB*	Bioscreen C	calibrated OD
52.5	126	51.78	BHIB*	Bioscreen C	calibrated OD
52.5	W16	57.91	BHIB*	Bioscreen C	calibrated OD
53	W16	52.80	BHIB*	Waterbaths	viable count
53	103A	45.00	BHIB*	Waterbaths	viable count
53	W5A	37.80	BHIB*	Waterbaths	viable count
53	126	87.60	BHIB*	Waterbaths	viable count
53	W7E	42.60	BHIB*	Waterbaths	viable count
53	T1C	127.20	BHIB*	Waterbaths	viable count
53	W16	45.00	BHIB*	Waterbaths	viable count
53	103A	42.60	BHIB*	Waterbaths	viable count
53	W5A	79.80	BHIB*	Waterbaths	viable count
53	W5B	75.00	BHIB*	Waterbaths	viable count
53	W14	52.80	BHIB*	Waterbaths	viable count
53	T80	77.40	BHIB*	Waterbaths	viable count
55	W14	29.40	BHIB*	Bioreactor	calibrated OD

Temp (°C)	Strain	Gen. Time (min.)	Growth Medium	Culture System	Method Used
55	W14	12.60	BHIB*	Bioreactor	viable count
55	50B	67.80	BHIB*	Waterbaths	viable count
55	W7E	112.80	BHIB*	Waterbaths	viable count
55	T1C	45.00	BHIB*	Waterbaths	viable count
55	W16	52.80	BHIB*	Waterbaths	viable count
55	291	92.40	BHIB*	Waterbaths	viable count
55	W7D	62.40	BHIB*	Waterbaths	viable count
55	W5A	57.60	BHIB*	Waterbaths	viable count
55	W5B	37.80	BHIB*	Waterbaths	viable count
55.5	50B	126.88	BHIB*	Bioscreen C	calibrated OD
55.5	W5A	101.90	BHIB*	Bioscreen C	calibrated OD
55.5	W14	83.49	BHIB*	Bioscreen C	calibrated OD
55.5	W5B	82.20	BHIB*	Bioscreen C	calibrated OD
55.5	W7E	78.13	BHIB*	Bioscreen C	calibrated OD
55.5	T1C	120.80	BHIB*	Bioscreen C	calibrated OD
55.5	103A	149.05	BHIB*	Bioscreen C	calibrated OD
55.5	W7D	98.33	BHIB*	Bioscreen C	calibrated OD
55.5	3D	120.52	BHIB*	Bioscreen C	calibrated OD
55.5	291	99.99	BHIB*	Bioscreen C	calibrated OD
55.5	T17	119.45	BHIB*	Bioscreen C	calibrated OD
55.5	W5D	87.91	BHIB*	Bioscreen C	calibrated OD
55.5	126	75.57	BHIB*	Bioscreen C	calibrated OD
55.5	W16	89.67	BHIB*	Bioscreen C	calibrated OD
57.5	50B	70.06	BHIB*	Bioscreen C	calibrated OD
57.5	W5A	40.60	BHIB*	Bioscreen C	calibrated OD
57.5	W14	51.18	BHIB*	Bioscreen C	calibrated OD
57.5	W5B	50.76	BHIB*	Bioscreen C	calibrated OD
57.5	W7E	55.49	BHIB*	Bioscreen C	calibrated OD
57.5	T1C	36.03	BHIB*	Bioscreen C	calibrated OD

Temp (°C)	Strain	Gen. Time (min.)	Growth Medium	Culture System	Method Used
57.5	103A	47.75	BHIB*	Bioscreen C	calibrated OD
57.5	W7D	41.63	BHIB*	Bioscreen C	calibrated OD
57.5	3D	170.78	BHIB*	Bioscreen C	calibrated OD
57.5	291	140.10	BHIB*	Bioscreen C	calibrated OD
57.5	T17	57.27	BHIB*	Bioscreen C	calibrated OD
57.5	W5D	52.47	BHIB*	Bioscreen C	calibrated OD
57.5	126	43.50	BHIB*	Bioscreen C	calibrated OD
57.5	W16	49.75	BHIB*	Bioscreen C	calibrated OD
58	T1C	21.00	BHIB*	Waterbaths	viable count
58	103A	42.00	BHIB*	Waterbaths	viable count
58	W5A	26.40	BHIB*	Waterbaths	viable count
58	W14	30.00	BHIB*	Waterbaths	viable count
58	T17	17.40	BHIB*	Waterbaths	viable count
58	28S	38.40	BHIB*	Waterbaths	viable count
58	T80	64.80	BHIB*	Waterbaths	viable count
58	50B	72.60	BHIB*	Waterbaths	viable count
58	3D	59.40	BHIB*	Waterbaths	viable count
58.5	50B	64.46	BHIB*	Bioscreen C	calibrated OD
58.5	W5A	51.14	BHIB*	Bioscreen C	calibrated OD
58.5	W14	67.98	BHIB*	Bioscreen C	calibrated OD
58.5	W5B	61.32	BHIB*	Bioscreen C	calibrated OD
58.5	W7E	68.16	BHIB*	Bioscreen C	calibrated OD
58.5	T1C	44.06	BHIB*	Bioscreen C	calibrated OD
58.5	103A	59.30	BHIB*	Bioscreen C	calibrated OD
58.5	W7D	50.74	BHIB*	Bioscreen C	calibrated OD
58.5	291	312.94	BHIB*	Bioscreen C	calibrated OD
58.5	T17	73.18	BHIB*	Bioscreen C	calibrated OD
58.5	W5D	77.05	BHIB*	Bioscreen C	calibrated OD
58.5	126	58.26	BHIB*	Bioscreen C	calibrated OD

Temp (°C)	Strain	Gen. Time (min.)	Growth Medium	Culture System	Method Used
58.5	W16	63.43	BHIB*	Bioscreen C	calibrated OD
59	50B	63.94	BHIB*	Bioscreen C	calibrated OD
59	W5A	56.91	BHIB*	Bioscreen C	calibrated OD
59	W14	60.11	BHIB*	Bioscreen C	calibrated OD
59	W5B	64.14	BHIB*	Bioscreen C	calibrated OD
59	W7E	71.83	BHIB*	Bioscreen C	calibrated OD
59	T1C	45.25	BHIB*	Bioscreen C	calibrated OD
59	103A	62.36	BHIB*	Bioscreen C	calibrated OD
59	W7D	50.82	BHIB*	Bioscreen C	calibrated OD
59	3D	121.78	BHIB*	Bioscreen C	calibrated OD
59	291	290.00	BHIB*	Bioscreen C	calibrated OD
59	T17	71.34	BHIB*	Bioscreen C	calibrated OD
59	W5D	91.92	BHIB*	Bioscreen C	calibrated OD
59	126	61.22	BHIB*	Bioscreen C	calibrated OD
59	W16	54.97	BHIB*	Bioscreen C	calibrated OD
59	126	30.60	BHIB*	Waterbaths	Double dilution
59	W7E	21.00	BHIB*	Waterbaths	Double dilution
59	T1C	28.80	BHIB*	Waterbaths	Double dilution
59	W16	25.80	BHIB*	Waterbaths	Double dilution
59	W7D	28.80	BHIB*	Waterbaths	Double dilution
59	103A	27.00	BHIB*	Waterbaths	Double dilution
59	W5A	33.00	BHIB*	Waterbaths	Double dilution
59	W5B	26.40	BHIB*	Waterbaths	Double dilution
59	W14	26.40	BHIB*	Waterbaths	Double dilution
59	T17	33.60	BHIB*	Waterbaths	Double dilution
59	T80	28.80	BHIB*	Waterbaths	Double dilution
59	50B	29.40	BHIB*	Waterbaths	Double dilution
59	3D	37.20	BHIB*	Waterbaths	Double dilution
60	W14	18.60	BHIB*	Bioreactor	viable count

Temp (°C)	Strain	Gen. Time (min.)	Growth Medium	Culture System	Method Used
60	W14	12.00	BHIB*	Bioreactor	viable count
60	W14	13.46	BHIB*	Waterbaths	viable count
60	W14	22.83	BHIB*	Waterbaths	viable count
60	W14	14.43	BHIB*	Waterbaths	viable count
60	W14	9.59	BHIB*	Waterbaths	viable count
60	W14	9.35	BHIB*	Waterbaths	viable count
60	W14	9.92	BHIB*	Waterbaths	viable count
61	W14	176.47	BHIB*	Waterbaths	viable count
61	W14	95.24	BHIB*	Waterbaths	viable count
63	W14	95.09	BHIB*	Waterbaths	viable count
63	W14	150.00	BHIB*	Waterbaths	viable count
63	W14	78.95	BHIB*	Waterbaths	viable count
65	W14	22.20	BHIB*	Bioreactor	viable count
65	W14	27.65	BHIB*	Bioreactor	viable count
65	W14	24.17	BHIB*	Waterbaths	viable count
65	W14	20.78	BHIB*	Waterbaths	viable count
65	W14	23.90	BHIB*	Waterbaths	viable count
65	W14	19.26	BHIB*	Waterbaths	viable count
65	W14	25.87	BHIB*	Waterbaths	viable count
65	W14	18.90	BHIB*	Waterbaths	viable count
70	W14	33.71	BHIB*	Bioreactor	viable count
70	W14	22.99	BHIB*	Bioreactor	viable count
70	W14	71.78	BHIB*	Waterbaths	viable count
70	W14	66.08	BHIB*	Waterbaths	viable count
70	W14	71.67	BHIB*	Waterbaths	viable count
70	W14	33.72	BHIB*	Waterbaths	viable count
70	W14	43.36	BHIB*	Waterbaths	viable count
70	W14	36.24	BHIB*	Waterbaths	viable count

* Brain Heart Infusion Broth

APPENDIX 2. Complete data set of strain, media used method system, temperature, showing SQRT GR, Fitted SR Rate, and SS including data not used for modelling.

Strain	Media	Method	Temp (°C)	SQRT GR	Fitted SR Rate	SS
126	SMB	Bioscreen	45	0.416776511	0.264499455	0.023188
291	SMB	Bioscreen	45	0.464332328	0.264499455	0.039933
103A	SMB	Bioscreen	45	0.112347584	0.264499455	0.02315
28S	SMB	Bioscreen	45	0.426250646	0.264499455	0.026163
3D	SMB	Bioscreen	45	0.291406882	0.264499455	0.000724
50B	SMB	Bioscreen	45	0.231185212	0.264499455	0.00111
T17	SMB	Bioscreen	45	0.16646781	0.264499455	0.00961
T1C	SMB	Bioscreen	45	0.179925208	0.264499455	0.007153
T80	SMB	Bioscreen	45	0.229616114	0.264499455	0.001217
W14	SMB	Bioscreen	45	0.133467065	0.264499455	0.017169
W16	SMB	Bioscreen	45	0.11381686	0.264499455	0.022705
W5A	SMB	Bioscreen	45	0.201640502	0.264499455	0.003951
W5B	SMB	Bioscreen	45	0.099437277	0.264499455	0.027246
W5D	SMB	Bioscreen	45	0.417190038	0.264499455	0.023314
W7D	SMB	Bioscreen	45	0.356527187	0.264499455	0.008469
W7E	SMB	Bioscreen	45	0.274166364	0.264499455	9.34E-05
W5A	BHI	Bioscreen	45	0.484855474	0.264499455	0.048557
W14	BHI	Bioscreen	45	0.3624966	0.264499455	0.009603
W5B	BHI	Bioscreen	45	0.372862679	0.264499455	0.011743
W7E	BHI	Bioscreen	45	0.386730774	0.264499455	0.01494
T1C	BHI	Bioscreen	45	0.209559145	0.264499455	0.003018
103A	BHI	Bioscreen	45	0.2978444	0.264499455	0.001112
W7D	BHI	Bioscreen	45	0.43853913	0.264499455	0.03029
3D	BHI	Bioscreen	45	0.218118626	0.264499455	0.002151
291	BHI	Bioscreen	45	0.206102195	0.264499455	0.00341
T17	BHI	Bioscreen	45	0.395755347	0.264499455	0.017228
W5D	BHI	Bioscreen	45	0.574637874	0.264499455	0.096186
26	BHI	Bioscreen	45	0.470724246	0.264499455	0.042529
W16	BHI	Bioscreen	45	0.391857819	0.264499455	0.01622
126	SMB	Bioscreen	45.5	0.13071551	0.290949243	0.025675
291	SMB	Bioscreen	45.5	0.129052468	0.290949243	0.026211
103A	SMB	Bioscreen	45.5	0.141678765	0.290949243	0.022282
28S	SMB	Bioscreen	45.5	0.279537918	0.290949243	0.00013
3D	SMB	Bioscreen	45.5	0.166363683	0.290949243	0.015522
50B	SMB	Bioscreen	45.5	0.161363101	0.290949243	0.016793
T17	SMB	Bioscreen	45.5	0.155759478	0.290949243	0.018276
T1C	SMB	Bioscreen	45.5	0.177626362	0.290949243	0.012842
W14	SMB	Bioscreen	45.5	0.145416304	0.290949243	0.02118

Strain	Media	Method	Temp (°C)	SQRT GR	Fitted SR Rate	SS
W16	SMB	Bioscreen	45.5	0.162481462	0.290949243	0.016504
W5A	SMB	Bioscreen	45.5	0.150009302	0.290949243	0.019864
W5B	SMB	Bioscreen	45.5	0.157532958	0.290949243	0.0178
W5D	SMB	Bioscreen	45.5	0.14949585	0.290949243	0.020009
W7D	SMB	Bioscreen	45.5	0.145706747	0.290949243	0.021095
W7E	SMB	Bioscreen	45.5	0.136535647	0.290949243	0.023844
W5A	BHI	Bioscreen	45.5	0.753711049	0.290949243	0.214148
W14	BHI	Bioscreen	45.5	0.706379188	0.290949243	0.172582
W5B	BHI	Bioscreen	45.5	0.754588177	0.290949243	0.214961
W7E	BHI	Bioscreen	45.5	0.675444436	0.290949243	0.147837
T1C	BHI	Bioscreen	45.5	0.582999619	0.290949243	0.085293
103A	BHI	Bioscreen	45.5	0.645946359	0.290949243	0.126023
W7D	BHI	Bioscreen	45.5	0.732982884	0.290949243	0.195394
3D	BHI	Bioscreen	45.5	0.669598329	0.290949243	0.143375
291	BHI	Bioscreen	45.5	0.763573235	0.290949243	0.223373
T17	BHI	Bioscreen	45.5	0.770578995	0.290949243	0.230045
W5D	BHI	Bioscreen	45.5	0.780772805	0.290949243	0.239927
126	BHI	Bioscreen	45.5	0.75402703	0.290949243	0.214441
W16	BHI	Bioscreen	45.5	0.665625105	0.290949243	0.140382
126	SMB	Bioscreen	47	0.336709546	0.370298057	0.001128
291	SMB	Bioscreen	47	0.416601833	0.370298057	0.002144
103A	SMB	Bioscreen	47	0.459169	0.370298057	0.007898
28S	SMB	Bioscreen	47	0.547549641	0.370298057	0.031418
3D	SMB	Bioscreen	47	0.529554164	0.370298057	0.025363
50B	SMB	Bioscreen	47	0.504620368	0.370298057	0.018042
T17	SMB	Bioscreen	47	0.479591472	0.370298057	0.011945
T1C	SMB	Bioscreen	47	0.480670235	0.370298057	0.012182
T80	SMB	Bioscreen	47	0.541911524	0.370298057	0.029451
W14	SMB	Bioscreen	47	0.385864014	0.370298057	0.000242
W16	SMB	Bioscreen	47	0.396766497	0.370298057	0.000701
W5A	SMB	Bioscreen	47	0.498112699	0.370298057	0.016337
W5B	SMB	Bioscreen	47	0.382755508	0.370298057	0.000155
W5D	SMB	Bioscreen	47	0.395299396	0.370298057	0.000625
W7D	SMB	Bioscreen	47	0.402922614	0.370298057	0.001064
W7E	SMB	Bioscreen	47	0.366525699	0.370298057	1.42E-05
50B	BHI	Bioscreen	47.5	0.700978592	0.396747369	0.092557
W5A	BHI	Bioscreen	47.5	0.881313488	0.396747369	0.234804
W14	BHI	Bioscreen	47.5	0.895425078	0.396747369	0.248679
W5B	BHI	Bioscreen	47.5	0.857631027	0.396747369	0.212414
W7E	BHI	Bioscreen	47.5	0.910198227	0.396747369	0.263632
T1C	BHI	Bioscreen	47.5	0.7717595	0.396747369	0.140634
103A	BHI	Bioscreen	47.5	0.820548338	0.396747369	0.179607
W7D	BHI	Bioscreen	47.5	0.772251952	0.396747369	0.141004
3D	BHI	Bioscreen	47.5	0.556915916	0.396747369	0.025654
291	BHI	Bioscreen	47.5	0.925354485	0.396747369	0.279425

Strain	Media	Method	Temp (°C)	SQRT GR	Fitted SR Rate	SS
T17	BHI	Bioscreen	47.5	0.871293422	0.396747369	0.225194
W5D	BHI	Bioscreen	47.5	0.893223946	0.396747369	0.246489
126	BHI	Bioscreen	47.5	0.938413962	0.396747369	0.293403
W16	BHI	Bioscreen	47.5	0.7691076	0.396747369	0.138652
126	SMB	Bioscreen	48	0.612269294	0.423196455	0.035749
291	SMB	Bioscreen	48	0.408650423	0.423196455	0.000212
103A	SMB	Bioscreen	48	0.520814851	0.423196455	0.009529
28S	SMB	Bioscreen	48	0.453893378	0.423196455	0.000942
3D	SMB	Bioscreen	48	0.453591649	0.423196455	0.000924
50B	SMB	Bioscreen	48	0.498993255	0.423196455	0.005745
T17	SMB	Bioscreen	48	0.199128876	0.423196455	0.050206
T1C	SMB	Bioscreen	48	0.320472259	0.423196455	0.010552
T80	SMB	Bioscreen	48	0.5389623	0.423196455	0.013402
W14	SMB	Bioscreen	48	0.465291767	0.423196455	0.001772
W16	SMB	Bioscreen	48	0.424351698	0.423196455	1.33E-06
W5A	SMB	Bioscreen	48	0.406065055	0.423196455	0.000293
W5B	SMB	Bioscreen	48	0.38140757	0.423196455	0.001746
W5D	SMB	Bioscreen	48	0.415015826	0.423196455	6.69E-05
W7D	SMB	Bioscreen	48	0.240602256	0.423196455	0.033341
W7E	SMB	Bioscreen	48	0.332226043	0.423196455	0.008276
126	SMB	Bioscreen	50	0.578829478	0.52898871	0.002484
291	SMB	Bioscreen	50	0.504511735	0.52898871	0.000599
103A	SMB	Bioscreen	50	0.599159666	0.52898871	0.004924
28S	SMB	Bioscreen	50	0.867889092	0.52898871	0.114853
3D	SMB	Bioscreen	50	0.736865978	0.52898871	0.043213
50B	SMB	Bioscreen	50	0.56643927	0.52898871	0.001403
T17	SMB	Bioscreen	50	0.524254628	0.52898871	2.24E-05
T1C	SMB	Bioscreen	50	0.601646928	0.52898871	0.005279
T80	SMB	Bioscreen	50	0.319299432	0.52898871	0.04397
W14	SMB	Bioscreen	50	0.630613636	0.52898871	0.010328
W16	SMB	Bioscreen	50	0.605001547	0.52898871	0.005778
W5A	SMB	Bioscreen	50	0.624270237	0.52898871	0.009079
W5B	SMB	Bioscreen	50	0.487970424	0.52898871	0.001682
W5D	SMB	Bioscreen	50	0.513543388	0.52898871	0.000239
W7D	SMB	Bioscreen	50	0.61878182	0.52898871	0.008063
W7E	SMB	Bioscreen	50	0.65883669	0.52898871	0.01686
126	SMB	Bioscreen	50.5	0.239970088	0.555435023	0.099518
291	SMB	Bioscreen	50.5	0.35107218	0.555435023	0.041764
103A	SMB	Bioscreen	50.5	0.284139272	0.555435023	0.073601
28S	SMB	Bioscreen	50.5	0.464982024	0.555435023	0.008182
3D	SMB	Bioscreen	50.5	0.425542914	0.555435023	0.016872
50B	SMB	Bioscreen	50.5	0.407480279	0.555435023	0.021891
T17	SMB	Bioscreen	50.5	0.217681929	0.555435023	0.114077
T1C	SMB	Bioscreen	50.5	0.277665415	0.555435023	0.077156
T80	SMB	Bioscreen	50.5	0.277735191	0.555435023	0.077117

Strain	Media	Method	Temp (°C)	SQRT GR	Fitted SR Rate	SS
W14	SMB	Bioscreen	50.5	0.242208678	0.555435023	0.098111
W16	SMB	Bioscreen	50.5	0.250355771	0.555435023	0.093073
W5A	SMB	Bioscreen	50.5	0.265726172	0.555435023	0.083931
W5B	SMB	Bioscreen	50.5	0.319338933	0.555435023	0.055741
W5D	SMB	Bioscreen	50.5	0.245250956	0.555435023	0.096214
W7D	SMB	Bioscreen	50.5	0.220112148	0.555435023	0.112441
W7E	SMB	Bioscreen	50.5	0.365887059	0.555435023	0.035928
50B	BHI	Bioscreen	50.5	0.626973862	0.555435023	0.005118
W5A	BHI	Bioscreen	50.5	0.866662414	0.555435023	0.096862
W14	BHI	Bioscreen	50.5	0.965037	0.555435023	0.167774
W5B	BHI	Bioscreen	50.5	1.074897938	0.555435023	0.269842
W7E	BHI	Bioscreen	50.5	0.958143903	0.555435023	0.162174
T1C	BHI	Bioscreen	50.5	0.885087316	0.555435023	0.108671
103A	BHI	Bioscreen	50.5	1.047529941	0.555435023	0.242157
W7D	BHI	Bioscreen	50.5	0.812484783	0.555435023	0.066075
3D	BHI	Bioscreen	50.5	0.482896101	0.555435023	0.005262
291	BHI	Bioscreen	50.5	0.97276923	0.555435023	0.174168
T17	BHI	Bioscreen	50.5	0.903777367	0.555435023	0.121342
W5D	BHI	Bioscreen	50.5	1.007024566	0.555435023	0.203933
126	BHI	Bioscreen	50.5	0.841018907	0.555435023	0.081558
W16	BHI	Bioscreen	50.5	0.842401331	0.555435023	0.08235
W16	Double dilution	BHI	51	1.5	0.581880177	0.842944
126	Double dilution	BHI	51	1	0.581880177	0.174824
126	SMB	Bioscreen	52	0.718603184	0.634765483	0.007029
291	SMB	Bioscreen	52	0.760404791	0.634765483	0.015785
103A	SMB	Bioscreen	52	0.730963351	0.634765483	0.009254
28S	SMB	Bioscreen	52	0.74789858	0.634765483	0.012799
3D	SMB	Bioscreen	52	0.482667496	0.634765483	0.023134
50B	SMB	Bioscreen	52	0.863616334	0.634765483	0.052373
T17	SMB	Bioscreen	52	0.671349655	0.634765483	0.001338
T1C	SMB	Bioscreen	52	0.658974388	0.634765483	0.000586
T80	SMB	Bioscreen	52	0.493292226	0.634765483	0.020015
W14	SMB	Bioscreen	52	0.789248655	0.634765483	0.023865
W16	SMB	Bioscreen	52	0.729976696	0.634765483	0.009065
W5A	SMB	Bioscreen	52	0.765125791	0.634765483	0.016994
W5B	SMB	Bioscreen	52	0.73892076	0.634765483	0.010848
W5D	SMB	Bioscreen	52	0.834371544	0.634765483	0.039843
W7D	SMB	Bioscreen	52	0.650106582	0.634765483	0.000235
W7E	SMB	Bioscreen	52	0.499191155	0.634765483	0.01838
126	SMB	Bioscreen	52.5	0.347482385	0.661204574	0.098422
291	SMB	Bioscreen	52.5	0.453737015	0.661204574	0.043043
103A	SMB	Bioscreen	52.5	0.446614768	0.661204574	0.046049
28S	SMB	Bioscreen	52.5	0.481468961	0.661204574	0.032305
3D	SMB	Bioscreen	52.5	0.515194476	0.661204574	0.021319
50B	SMB	Bioscreen	52.5	0.487907298	0.661204574	0.030032

Strain	Media	Method	Temp (°C)	SQRT GR	Fitted SR Rate	SS
T17	SMB	Bioscreen	52.5	0.396888757	0.661204574	0.069863
T1C	SMB	Bioscreen	52.5	0.530808746	0.661204574	0.017003
T80	SMB	Bioscreen	52.5	0.476865606	0.661204574	0.033981
W14	SMB	Bioscreen	52.5	0.367226008	0.661204574	0.086423
W16	SMB	Bioscreen	52.5	0.357111322	0.661204574	0.092473
W5A	SMB	Bioscreen	52.5	0.319603933	0.661204574	0.116691
W5B	SMB	Bioscreen	52.5	0.350876704	0.661204574	0.096303
W5D	SMB	Bioscreen	52.5	0.411900194	0.661204574	0.062153
W7D	SMB	Bioscreen	52.5	0.343436378	0.661204574	0.100977
W7E	SMB	Bioscreen	52.5	0.429350055	0.661204574	0.053757
50B	BHI	Bioscreen	52.5	0.757720975	0.661204574	0.009315
W5A	BHI	Bioscreen	52.5	1.050861145	0.661204574	0.151832
W14	BHI	Bioscreen	52.5	0.99721764	0.661204574	0.112905
W5B	BHI	Bioscreen	52.5	1.108369437	0.661204574	0.199956
W7E	BHI	Bioscreen	52.5	1.166459646	0.661204574	0.255283
T1C	BHI	Bioscreen	52.5	1.050967962	0.661204574	0.151915
103A	BHI	Bioscreen	52.5	1.06629636	0.661204574	0.164099
W7D	BHI	Bioscreen	52.5	0.95842995	0.661204574	0.088343
3D	BHI	Bioscreen	52.5	0.916219301	0.661204574	0.065033
291	BHI	Bioscreen	52.5	1.184828041	0.661204574	0.274182
T17	BHI	Bioscreen	52.5	1.091470595	0.661204574	0.185129
W5D	BHI	Bioscreen	52.5	1.069552155	0.661204574	0.166748
126	BHI	Bioscreen	52.5	1.076427838	0.661204574	0.17241
W16	BHI	Bioscreen	52.5	1.017875281	0.661204574	0.127214
W16	Double dilution	BHI	53	0.88	0.687640313	0.037002
103A	Double dilution	BHI	53	0.75	0.687640313	0.003889
W5A	Double dilution	BHI	53	0.63	0.687640313	0.003322
126	Double dilution	BHI	53	1.46	0.687640313	0.596539
W7E	Double dilution	BHI	53	0.71	0.687640313	0.0005
T1C	Double dilution	BHI	53	2.12	0.687640313	2.051654
W16	Double dilution	BHI	53	0.75	0.687640313	0.003889
103A	Double dilution	BHI	53	0.71	0.687640313	0.0005
W5B	Double dilution	BHI	53	1.25	0.687640313	0.316248
W14	Double dilution	BHI	53	0.88	0.687640313	0.037002
T80	Double dilution	BHI	53	1.29	0.687640313	0.362837
W14	Skim milk	Bioreactor	55	1.428571429	0.79332454	0.403539
W14	Skim milk	Bioreactor	55	2.182178902	0.79332454	1.928916
50B	BHI	Bioscreen	55	0.687677834	0.79332454	0.011161
W5A	BHI	Bioscreen	55	0.767353614	0.79332454	0.000674
W14	BHI	Bioscreen	55	0.84771862	0.79332454	0.002959
W5B	BHI	Bioscreen	55	0.8543746	0.79332454	0.003727
W7E	BHI	Bioscreen	55	0.876354045	0.79332454	0.006894
T1C	BHI	Bioscreen	55	0.704751245	0.79332454	0.007845
103A	BHI	Bioscreen	55	0.634464131	0.79332454	0.025237
W7D	BHI	Bioscreen	55	0.781148174	0.79332454	0.000148

Strain	Media	Method	Temp (°C)	SQRT GR	Fitted SR Rate	SS
3D	BHI	Bioscreen	55	0.705593562	0.79332454	0.007697
291	BHI	Bioscreen	55	0.774619572	0.79332454	0.00035
T17	BHI	Bioscreen	55	0.708728805	0.79332454	0.007156
W5D	BHI	Bioscreen	55	0.826128813	0.79332454	0.001076
126	BHI	Bioscreen	55	0.891038932	0.79332454	0.009548
W16	BHI	Bioscreen	55	0.817985498	0.79332454	0.000608
50B	Double dilution	BHI	55	1.13	0.79332454	0.11335
W7E	Double dilution	BHI	55	1.88	0.79332454	1.180864
T1C	Double dilution	BHI	55	0.75	0.79332454	0.001877
W16	Double dilution	BHI	55	0.88	0.79332454	0.007513
291	Double dilution	BHI	55	1.54	0.79332454	0.557524
W7D	Double dilution	BHI	55	1.04	0.79332454	0.060849
W5A	Double dilution	BHI	55	0.96	0.79332454	0.027781
W5B	Double dilution	BHI	55	0.63	0.79332454	0.026675
126	SMB	Bioscreen	55.5	0.461869983	0.819720937	0.128057
291	SMB	Bioscreen	55.5	0.555096443	0.819720937	0.070026
103A	SMB	Bioscreen	55.5	0.519917587	0.819720937	0.089882
28S	SMB	Bioscreen	55.5	0.611125476	0.819720937	0.043512
3D	SMB	Bioscreen	55.5	0.73682717	0.819720937	0.006871
50B	SMB	Bioscreen	55.5	0.479847531	0.819720937	0.115514
T17	SMB	Bioscreen	55.5	0.548881018	0.819720937	0.073354
T1C	SMB	Bioscreen	55.5	0.57159367	0.819720937	0.061567
W14	SMB	Bioscreen	55.5	0.424830752	0.819720937	0.155938
W16	SMB	Bioscreen	55.5	0.725033571	0.819720937	0.008966
W5A	SMB	Bioscreen	55.5	0.499583399	0.819720937	0.102488
W5B	SMB	Bioscreen	55.5	0.482142401	0.819720937	0.113959
W5D	SMB	Bioscreen	55.5	0.597472453	0.819720937	0.049394
W7D	SMB	Bioscreen	55.5	0.558656249	0.819720937	0.068155
W7E	SMB	Bioscreen	55.5	0.597492377	0.819720937	0.049386
126	SMB	Bioscreen	57.5	0.497274472	0.925091108	0.183027
291	SMB	Bioscreen	57.5	0.747452434	0.925091108	0.031555
103A	SMB	Bioscreen	57.5	0.618739425	0.925091108	0.093851
28S	SMB	Bioscreen	57.5	0.746326421	0.925091108	0.031957
3D	SMB	Bioscreen	57.5	0.545382194	0.925091108	0.144179
50B	SMB	Bioscreen	57.5	0.6194539	0.925091108	0.093414
T17	SMB	Bioscreen	57.5	0.521487695	0.925091108	0.162896
T1C	SMB	Bioscreen	57.5	1.047533802	0.925091108	0.014992
T80	SMB	Bioscreen	57.5	0.609141977	0.925091108	0.099824
W14	SMB	Bioscreen	57.5	0.529244691	0.925091108	0.156694
W16	SMB	Bioscreen	57.5	0.410135707	0.925091108	0.265179
W5A	SMB	Bioscreen	57.5	0.633934779	0.925091108	0.084772
W5B	SMB	Bioscreen	57.5	0.564177037	0.925091108	0.130259
W5D	SMB	Bioscreen	57.5	0.571674749	0.925091108	0.124903
W7D	SMB	Bioscreen	57.5	0.50930811	0.925091108	0.172876
W7E	SMB	Bioscreen	57.5	0.629650338	0.925091108	0.087285

Strain	Media	Method	Temp (°C)	SQRT GR	Fitted SR Rate	SS
50B	BHI	Bioscreen	57.5	0.925397802	0.925091108	9.41E-08
W5A	BHI	Bioscreen	57.5	1.215608319	0.925091108	0.0844
W14	BHI	Bioscreen	57.5	1.08269506	0.925091108	0.024839
W5B	BHI	Bioscreen	57.5	1.087191139	0.925091108	0.026276
W7E	BHI	Bioscreen	57.5	1.039826397	0.925091108	0.013164
T1C	BHI	Bioscreen	57.5	1.290429541	0.925091108	0.133472
103A	BHI	Bioscreen	57.5	1.120941024	0.925091108	0.038357
W7D	BHI	Bioscreen	57.5	1.200531704	0.925091108	0.075868
3D	BHI	Bioscreen	57.5	0.592725625	0.925091108	0.110467
291	BHI	Bioscreen	57.5	0.654422586	0.925091108	0.073261
T17	BHI	Bioscreen	57.5	1.023551562	0.925091108	0.009694
W5D	BHI	Bioscreen	57.5	1.069362899	0.925091108	0.020814
126	BHI	Bioscreen	57.5	1.174381118	0.925091108	0.062146
W16	BHI	Bioscreen	57.5	1.098218496	0.925091108	0.029973
T1C	Double dilution	BHI	58	0.35	0.951343777	0.361614
103A	Double dilution	BHI	58	0.7	0.951343777	0.063174
W5A	Double dilution	BHI	58	0.44	0.951343777	0.261472
W14	Double dilution	BHI	58	0.5	0.951343777	0.203711
T17	Double dilution	BHI	58	0.29	0.951343777	0.437376
28S	Double dilution	BHI	58	0.64	0.951343777	0.096935
T80	Double dilution	BHI	58	1.08	0.951343777	0.016552
50B	Double dilution	BHI	58	1.21	0.951343777	0.066903
3D	Double dilution	BHI	58	0.99	0.951343777	0.001494
50B	BHI	Bioscreen	58.5	0.964784545	0.977538207	0.000163
W5A	BHI	Bioscreen	58.5	1.083176719	0.977538207	0.011159
W14	BHI	Bioscreen	58.5	0.939443364	0.977538207	0.001451
W5B	BHI	Bioscreen	58.5	0.989140998	0.977538207	0.000135
W7E	BHI	Bioscreen	58.5	0.938265605	0.977538207	0.001542
T1C	BHI	Bioscreen	58.5	1.166910947	0.977538207	0.035862
103A	BHI	Bioscreen	58.5	1.005896512	0.977538207	0.000804
W7D	BHI	Bioscreen	58.5	1.087387842	0.977538207	0.012067
291	BHI	Bioscreen	58.5	0.43786665	0.977538207	0.291245
T17	BHI	Bioscreen	58.5	0.905495573	0.977538207	0.00519
W5D	BHI	Bioscreen	58.5	0.882435181	0.977538207	0.009045
126	BHI	Bioscreen	58.5	1.014861098	0.977538207	0.001393
W16	BHI	Bioscreen	58.5	0.97257705	0.977538207	2.46E-05
126	SMB	Bioscreen	59	0.460040131	1.003657407	0.29552
291	SMB	Bioscreen	59	0.599078018	1.003657407	0.163684
103A	SMB	Bioscreen	59	0.536846028	1.003657407	0.217913
28S	SMB	Bioscreen	59	0.722035038	1.003657407	0.079311
3D	SMB	Bioscreen	59	0.678050162	1.003657407	0.10602
50B	SMB	Bioscreen	59	0.721692341	1.003657407	0.079504
T17	SMB	Bioscreen	59	0.710822918	1.003657407	0.085752
T1C	SMB	Bioscreen	59	0.735671854	1.003657407	0.071816
T80	SMB	Bioscreen	59	0.667403714	1.003657407	0.113067

Strain	Media	Method	Temp (°C)	SQRT GR	Fitted SR Rate	SS
W14	SMB	Bioscreen	59	0.523513443	1.003657407	0.230538
W16	SMB	Bioscreen	59	0.5220546	1.003657407	0.231941
W5A	SMB	Bioscreen	59	0.500195383	1.003657407	0.253474
W5B	SMB	Bioscreen	59	0.701711831	1.003657407	0.091171
W5D	SMB	Bioscreen	59	0.481591928	1.003657407	0.272552
W7D	SMB	Bioscreen	59	0.480166376	1.003657407	0.274043
W7E	SMB	Bioscreen	59	0.451695922	1.003657407	0.304661
50B	BHI	Bioscreen	59	0.968681879	1.003657407	0.001223
W5A	BHI	Bioscreen	59	1.026824667	1.003657407	0.000537
W14	BHI	Bioscreen	59	0.999048066	1.003657407	2.12E-05
W5B	BHI	Bioscreen	59	0.967224429	1.003657407	0.001327
W7E	BHI	Bioscreen	59	0.913952002	1.003657407	0.008047
T1C	BHI	Bioscreen	59	1.151512457	1.003657407	0.021861
103A	BHI	Bioscreen	59	0.98091964	1.003657407	0.000517
W7D	BHI	Bioscreen	59	1.08656146	1.003657407	0.006873
3D	BHI	Bioscreen	59	0.701924634	1.003657407	0.091043
291	BHI	Bioscreen	59	0.454861567	1.003657407	0.301177
T17	BHI	Bioscreen	59	0.91711352	1.003657407	0.00749
W5D	BHI	Bioscreen	59	0.80793733	1.003657407	0.038306
126	BHI	Bioscreen	59	0.990000976	1.003657407	0.000186
W16	BHI	Bioscreen	59	1.044746644	1.003657407	0.001688
126	Double dilution	BHI	59	0.51	1.003657407	0.243698
W7E	Double dilution	BHI	59	0.35	1.003657407	0.427268
T1C	Double dilution	BHI	59	0.48	1.003657407	0.274217
W16	Double dilution	BHI	59	0.43	1.003657407	0.329083
W7D	Double dilution	BHI	59	0.48	1.003657407	0.274217
103A	Double dilution	BHI	59	0.45	1.003657407	0.306537
W5A	Double dilution	BHI	59	0.55	1.003657407	0.205805
W5B	Double dilution	BHI	59	0.44	1.003657407	0.31771
W14	Double dilution	BHI	59	0.44	1.003657407	0.31771
T17	Double dilution	BHI	59	0.56	1.003657407	0.196832
T80	Double dilution	BHI	59	0.48	1.003657407	0.274217
50B	Double dilution	BHI	59	0.49	1.003657407	0.263844
3D	Double dilution	BHI	59	0.62	1.003657407	0.147193
W14	Skim milk	Bioreactor	60	1.79605302	1.055576202	0.548306
W14	Skim milk	Bioreactor	60	2.236067977	1.055576202	1.393561
W14	Waterbath	BHI	60	2.111273332	1.055576202	1.114496
T17	Waterbath	BHI	60	1.43682004	1.055576202	0.145347
W7D	Waterbath	BHI	60	0.892381933	1.055576202	0.026632
W14	Waterbath	BHI	60	2.613852695	1.055576202	2.428226
T17	Waterbath	BHI	60	2.54794556	1.055576202	2.227166
W7D	Waterbath	BHI	60	2.632091749	1.055576202	2.485401
W14	Waterbath	BHI	61	0.583095189	1.106837796	0.274306
W16	Waterbath	BHI	61	0.793725393	1.106837796	0.098039
126	Waterbath	BHI	63	0.794355084	1.205364974	0.168929

Strain	Media	Method	Temp (°C)	SQRT GR	Fitted SR Rate	SS
W14	Waterbath	SMB	63	0.632455532	1.205364974	0.328225
126	Waterbath	SMB	63	0.871779789	1.205364974	0.111279
W14	Skim milk	Bioreactor	65	1.643989873	1.29111032	0.124524
W14	Skim milk	Bioreactor	65	1.473091986	1.29111032	0.033117
W14	Waterbath	BHI	65	2.10938419	1.29111032	0.669572
W14	Waterbath	BHI	65	1.180123311	1.29111032	0.012318
T17	Waterbath	BHI	65	1.417498616	1.29111032	0.015974
W7D	Waterbath	BHI	65	1.510648801	1.29111032	0.048197
W14	Waterbath	BHI	65	2.033441349	1.29111032	0.551055
T17	Waterbath	BHI	65	2.347261074	1.29111032	1.115454
W7D	Waterbath	BHI	65	1.414213562	1.29111032	0.015154
W14	Skim milk	Bioreactor	70	1.334176186	1.196744864	0.018887
W14	Skim milk	Bioreactor	70	1.615615008	1.196744864	0.175452
W14	Waterbath	BHI	70	0.936168767	1.196744864	0.0679
T17	Waterbath	BHI	70	1.417498616	1.196744864	0.048732
W7D	Waterbath	BHI	70	0.87868824	1.196744864	0.10116
W14	Waterbath	BHI	70	0.952004355	1.196744864	0.059898
T17	Waterbath	BHI	70	1.254625992	1.196744864	0.00335
W7D	Waterbath	BHI	70	0.67218096	1.196744864	0.275167

APPENDIX 3. The complete data set of the growth of *G. stearothermophilus* W14 generated using the flow-through reactor system.

Method of flow-through experiments	Reference	Reference	Temperature (°C)	VEGETATIVE CELLS	SPORES	VEG CELLS - SPORES (difference)							
				GT (min)	GT (h)	SQRT GR (h)	LAG (h)	RLT	GT (min)	GT (h)	LAG (h)	Average (Log CFU/mL)	standard dev
Continuous (Pre-Modelled)	Exp#1	1	50	61	1.02	0.99	2.5	2.5	131	2.18	11.9	2.56	0.45
Continuous (Pre-Modelled)	Exp#2	2	55	71	1.19	0.92	1.5	1.2	63	1.05	11.3	2.58	0.56
Continuous (Pre-Modelled)	Exp#3	3	55	34	0.56	1.34	3.6	6.5	n/a	ng	ng	n/a	n/a
Continuous	Exp#8	4	45	27	0.45	1.48	16.1	35.3	n/a	ng	ng	n/a	n/a
Continuous	Exp#10	5	50	77	1.28	0.88	7.5	5.9	n/a	n/a	>20	2.8	0.99
Continuous	Exp#4	6	55	38	0.64	1.25	5.0	7.9	32	0.53	1.9	2.97	1.05
Continuous	Exp#7	7	65	72	1.19	0.92	2.9	2.4	77	1.28	5.3	1.90	0.46
Continuous	Exp#5	8	65	43	0.71	1.19	1.7	2.4	37	0.62	5.5	2.86	0.27
Continuous	Exp#6	9	65	24	0.40	1.59	2.6	6.6	25	0.41	7.3	3.30	0.44
Continuous	Exp#11	10	70	0	n/a	n/a	>24	n/a	n/a	ng	ng	n/a	n/a
Continuous	Exp#9	11	75	n/a	ng	n/a	n/a	n/a	n/a	n/a	≥24	n/a	n/a
Pulsed	Exp#13	12	55	0	n/a	n/a	≥22	n/a	n/a	ng	ng	n/a	n/a
Pulsed	Exp#16	13	55	60	0.99	1.00	5.2	5.2	n/a	ng	>24	n/a	n/a
Pulsed	Exp#12	14	60	29	0.49	1.44	14.4	29.7	n/a	n/a	>21	n/a	n/a
Pulsed	Exp#15	15	65	42	0.71	1.19	1.6	2.2	0	n/a	6.8	2.15	1.76
Pulsed	Exp#14	16	67.5	35	0.59	1.30	8.7	14.7	40	0.67	4.1	3.75	0.43

n/a = not applicable; ng = no growth

APPENDIX 4. Sample Preparation for Electron Microscopy

This method is taken from Nilsson (2010).

After incubation, stainless steel coupons were removed from the medium and rinsed 5 times using 10 mL of 1 × phosphate buffered saline (pH 7.4). The coupons were placed into 0.2 M cacodylate buffer (pH 7.4) containing 25mL/L glutaraldehyde (Proscitech, Brisbane, Australia) for 24 hours for primary fixation. Following primary fixation, the coupons were given two 5-min washes in 10 mL of cacodylate buffer supplemented with 6% sucrose and 0.1% calcium chloride (pH 7.4) and placed into 1% osmium tetroxide for 20 minutes. The coupons then underwent a graded series of ethanol washes (15 minutes each at 40, 50, 70, 90 and 100% ethanol). Immediately after the final wash, the coupons were freeze-dried using a vacuum freeze dryer (Dynavac, U.S.A.) for 5 hours. The dried coupons were sputter coated with gold using a BalTec SCD 050 sputter coater, and viewed with an Hitachi SU-70 Ultra High Resolution Schottky environmental scanning electron microscope operated at an accelerating voltage of 15kV.

A4.1 Preparation of Chemicals

A4.1.1 Osmium Tetroxide (1%)

5 mL 4 % Osmium tetroxide solution (ProSciTech, Queensland, Australia)

15 mL Single distilled water

Osmium tetroxide is highly toxic. All steps were performed in a fume hood and

wearing protective clothing. All of the ingredients were combined and mixed gently.

A4.1.2 Phosphate Buffered Saline (10× stock)

80 g Sodium chloride (NaCl) (Sigma - Aldrich, Castle Hill, Australia)

2 g Potassium chloride (KCl) (Sigma - Aldrich, Castle Hill, Australia)

2.4 g Sodium phosphate (KH_2PO_4) (Sigma - Aldrich, Castle Hill, Australia)

1000 mL Single distilled water

All ingredients were combined and dissolved by stirring using a magnetic stirrer before autoclaving at 121°C for 20 minutes. The 10 × stock has a pH of ≈ 6.8. When diluted to 1× the pH adjusts to ≈ 7.4. Fine adjustments were made using hydrochloric acid or sodium hydroxide.

A4.1.3 Cacodylate Buffer Stock Solution (0.2 M)

20.15 g Sodium cacodylate trihydrate (ProSciTech, Queensland, Australia)

0.1 mL Hydrochloric acid (glacial) (Sigma - Aldrich, Castle Hill, Australia)

250 mL Single distilled water

Cacodylate is a known carcinogen. All steps were performed in a fume hood and wearing appropriate protective clothing. pH was adjusted to 7.4 if necessary using hydrochloric acid or sodium hydroxide.

A4.1.4 Cacodylate Buffer with 6% Sucrose and 0.1% Calcium Chloride (0.1 M)

250 mL 0.2 M cacodylate stock solution (A3.1.3)

30 g RNAase free sucrose (Sigma - Aldrich, Castle Hill, Australia)

0.05 g Calcium chloride (Sigma - Aldrich, Castle Hill, Australia)

250 mL Single distilled water

Cacodylate is a known carcinogen. All steps were performed in a fume hood and wearing appropriate protective clothing. pH was adjusted to 7.4 if necessary using hydrochloric acid or sodium hydroxide. All ingredients were combined and gently mixed.